THE USE OF A BIOLUMINESCENT MARKER TO EVALUATE ESCHERICHIA COLI O157:H7 SUBLETHAL INJURY AND CROSS PROTECTION IN APPLE CIDER

By

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The primary objectives of this research included: 1.) Assessing the presence of sublethal injury by observing nonculturable E. coli O157:H7 after employing preservation methods and 2.) Determining if E. coli O157:H7, that have been cultured in acidified media, show cross protection or increased resistance to certain preservation methods. These objectives were achieved by using luminometric methods to enumerate organisms that were transformed with bioluminescent markers. Three environmental strains of E. coli O157:H7 were electrotransformed using plasmid DNA (pT7-5) that codes for bioluminescence. Correlation between plate counts (CFU/mL) and bioluminescence, measured as relative light units (RLU), were established for all experimental conditions using linear regression analysis. The transformants were cultured in acidified (pH 5.0) growth media to prepare acid adapted cells or grown in neutral media to provide nonadapted cells. These conditioned E. coli were subjected to benzoate/sorbate (0.1%), freeze (-20°C) - thawing (4°C for 4 hours), and thermal (54°C) treatments and enumerated by standard

plate count and luminometry. Strain-specific survival behavior was evaluated by comparing the effect of the treatments on each strain according to counts obtained. Sublethal injury was measured by obtaining a significant ($\alpha = 0.05$) positive difference after subtracting plate count D-values, representing culturable bacteria, from RLU-derived D-values, which represent the total viable bacteria. Cross protection was assessed by observing ($\alpha = 0.05$) significantly higher mean D-values for acid-adapted cells than mean D-values for nonadapted cells.

It was determined in this study that variation between strains existed after each treatment. Benzoate- and sorbate-treated acid-adapted E. coli O157:H7 showed between a 1.5 to 3-log reduction depending on the strain. E. coli O157:H7 strains showed cell count declines ranging from 3 to 5.5-logs after freeze-thaw treatment. After thermal treatment, E. coli O157:H7 cells declined between 3.5 to 7-logs. Significant sublethal injury was apparent in all matrices, especially after freeze-thaw treatment. Significant ($\alpha = 0.05$) cross protection existed after each treatment by most strains used in the study, except for freeze-thaw treated cells. This study validates the use of bioluminescence-marked E. coli O157:H7 for studying cross protection and sublethal injury.

INTRODUCTION

Unlike the commensal, non-diarrheogenic members of Enterobacteriaceae, E. coli O157:H7 has received much attention concerning the diarrheogenic properties associated with the serotype. First implicated in 1982 as the causative agent in 3 North American outbreaks, the organism is now believed to be responsible for 20,000 infections and 250 deaths per year (Boyce et al., 1995). E. coli O157:H7 categorized as enterohemorragic (EHEC), is unique from other E. coli with respect to biochemical, pathogenic, clinical, and epidemiological characteristics. Its inability to ferment sorbitol and to hydrolyze methylumbelliferyl β-D-glucuronide (MUG) allow E. coli O157:H7 to be distinguished from most other E. coli through isolation techniques. Virulence factors associated with E. coli O157:H7, including attachment factors and cytotoxins, have been characterized heavily (Jackson et al., 1987; Strockbine, 1988). These factors contribute to the clinical manifestations of E. coli O157:H7 that range from mild self-limiting symptoms to more serious renal and neurological diseases that are sometimes fatal. Incidence of these diseases is worldwide but the diseases have been most prevalent in North America. Other data indicate that seasonal and age factors, as well as vehicles of transmission, play an important role with respect to the epidemiology associated with the organism. E. coli O157 is perhaps most notorious for being linked with improperly cooked ground beef, but E. coli O157:H7 outbreaks from other food and non-food sources have also been significant. Therefore, accurate E. coli O157:H7 detection and enumeration techniques have been valuable in reducing the risk of disease. These techniques range from conventional culturing methods to those involving rapid E. coli O157:H7

specific serological and DNA-oriented protocols. Several studies have used techniques such as these to identify survival features related to E. coli O157:H7.

Recent studies have illustrated that E. coli O157:H7 and other members of enterobacteriaceae are able to survive and grow in acidic environments. This feature is not fully understood but studies suggest that survival at low pH (pH 5 to 6) involves cell homeostasis responses. Cell homeostasis is achieved, in part, by the regulation of proton influx by a membrane-bound proton pump gradient (Mitchell, 1973). However, the effectiveness of this pump is limited at extremely low pH (< 4.0). Survival in extreme acidic situations (pH 3 to 4) is known to occur after prehabituation to moderately low pH (pH 5). This preexposure is believed to initiate an acidification tolerance response (ATR) involving the generation of proteins that protect the organism from proton damage (Raja et al., 1991). The proposed protective functions of these proteins include stabilizing the outer membrane and buffering the impact of protons through decarboxylation of the amino acids. Strong evidence suggests that protein generation and other protective responses are a function of a stress-induced sigma factor designated as rpoS. Initiation of the rpoS regulatory operon is influenced by arginine- and glutamate-dependent systems according to scientists (Lin et al, 1996).

The protective responses initiated by bacteria after pre-exposure to low pH and other stress factors are known to enhance survival under additional forms of pressure. This phenomenon referred to as cross protection has been studied to allow resistance to factors including heat, sodium chloride, and osmolarity changes. The exact mechanisms behind cross protection are highly controversial and are

hypothesized to be the result of a combination of events including protein synthesis. Proteins that are generated are suspected to stabilize the outer membrane or to increase cell surface hydrophobicity of the cell (Leyer and Johnson, 1992). These measures may allow the cell to survive treatment with compounds that would otherwise be immediately deleterious to the organism.

Microorganisms that survive harsh antimicrobial treatments may be incapacitated temporarily and unable to multiply. These sublethally injured organisms eventually recover from damage to various intracellular and extracellular target sites including the cell wall, cytoplasmic membrane, and nucleic acids. In efforts to better understand sublethal injury, several techniques have been employed to measure for their existence. Traditional techniques involve obtaining plate count differences from cells that have been plated on restrictive and optimum media after stress treatment. More advanced techniques involve using a flow cytometer in combination with subjecting the cells to fluorogenic and chromogenic compounds. Through flow cytometry, sublethally injured cells are rapid but sometimes not accurate.

Another method of measuring sublethally injured cells involves transforming the organism of interest with a bioluminescent marker. The bioluminescent reaction is a function of the luciferase (*lux*) gene, which is typically isolated from marine halophiles. The enzyme luciferase produces bioluminescence using the reduced form of flavin mononucleotide generated by the viable cell. The bioluminescence system has proven to be very effective in measuring viable cell counts in several studies.

This system may be useful to the food industry, as it will allow for more accurate assessment of the efficacy of their intervention strategies.

Because of recent fresh-juice-associated outbreaks, the fresh juice industry and regulatory agencies are concerned with microbial control strategies (CDC, 1976; 1982; 1996; 1997). Currently, the Food and Drug Administration (FDA) supports a warning label requirement, a safety control program, and microbial-reduction measures that affect all processors that do not treat their fresh juices and ciders to achieve a five-log reduction. Alternatives to pasteurization have been pursued in efforts to comply with the seemingly imminent microbial control requirements. These alternatives include benzoate/sorbate, freeze-thaw, and mild thermal treatment.

Pending regulatory activity has made studies into effectiveness intervention strategies even more important, as fresh juice and cider manufacturers will be forced to seek alternative microbial-control programs. A quick accurate means of performing such analyses may involve the bioluminescent system. This system will allow strain characterizations to be made, will take into account sublethal injury, and will evaluate degree of cross protection. The bioluminescence system has not been used in cross protection studies.

The objectives in this study include: 1.) Succesfully transforming three environmental strains E. coli O157:H7 using the pT7-5 plasmid conferring bioluminescence 2.) Develop linear regression models that correlate ($R^2 > 0.95$) RLU with CFU in buffer, minimal media, and apple cider. 3.) Evaluate the effects of benzoate/sorbate, freeze-thaw, and thermal treatments on the environmental strains. 4.) Assess sublethal injury by statistically comparing ($\alpha = 0.05$) D-values obtained

from plate counts, with D-values derived from using relative light unit measurements.

5.) Evaluate cross protection statistically comparing ($\alpha = 0.05$) D-values of acid-adapted to those that were nonadapted. Based on data obtained from previous studies, it is expected that sublethal injury and cross protection will be observed.

LITERATURE REVIEW

E. coli 0157:H7 Bacteria

The foodborne bacterial pathogen E. coli O157:H7 is considered a major public health concern in the United States, responsible for an estimated 10,000 to 20,000 infections and 250 deaths per year (Boyce et al., 1995). It is also believed to be associated with hundreds of thousands of unreported sporadic cases throughout the world (Tarr et al., 1990). E. coli O157:H7 first emerged as a significant pathogen in 1982, when it was implicated as the causative agent in 3 major North American outbreaks. Ever since these outbreaks, major efforts have been made to characterize the organism according to its microbiological, pathological, clinical, and epidemiological features. Distinctions based on these characteristics have allowed scientists to detect and enumerate E. coli O157:H7 found in foods and other sources (Wells et al., 1983; CDC, 1982; Riley et al., 1983).

Microbiology

Escherichia coli, a member of the family enterobacteriaceae, is a facultative anaerobic, gram-negative bacillus that usually contains peritrichous flagella for motility. The O157:H7 designation refers to the serotypic characteristics of E. coli, with O and H representing surface-oriented somatic and flagellar antigens, respectively. E. coli O157:H7 differs from commensal, nondiarrheogenic E. coli residing in the intestinal tract of warm-blooded hosts as it displays pathogenic

properties after infection and is therefore classified as diarrheogenic. E. coli O157:H7 is distinguished from other diarrheogenic E. coli including those grouped as enteropathogenic (EPEC), enterotoxigenic (ETEC), enteroinvasive (EIEC), enteroaggregative (EAggEC), and diffuse adhering (DAEC), because E. coli O157:H7 produces unique cytotoxins. These cytotoxins are able to disrupt Vero cells, isolated from green monkeys. Hence, E. coli O157:H7 is grouped as Vero cytotoxin-producing E. coli (VTEC). Although nonpathogenic VTEC E. coli exist, E. coli O157:H7, designated as enterohemmhorragic E. coli (EHEC), maintains other virulence factors contributing to hemmhorrage formation in host intestines.

The main biochemical features shared by all E. coli are the inability to generate oxidase and the ability to ferment glucose to produce acid and gas. E. coli O157:H7 is distinguished from most E. coli with respect to sorbitol fermentation and β-glucuronidase activity. Nearly all E. coli (93%) ferment sorbitol within a 24-hour period while E. coli O157:H7 does not show clear colony formation when cultured on sorbitol-MacConkey agar (Wells et al., 1983). Also, 93% of E. coli generate β-glucuronidase, an enzyme that hydrolyzes the substrate 4-methylumbelliferyl β-D-glucuronide (MUG) to exhibit flourescence after exposure to ultraviolet light. E. coli O157:H7 is unable to produce this enzyme and therefore does not display fluorescence.

E. coli bacteria generally grow at a wide temperature range between 15°C and 45°C. Unlike generic E. coli, E. coli O157:H7 strains typically only grows between 30 C and 42°C and show very poor growth (if any) at temperatures between 44°C and 45°C. Currently implemented screening procedures take advantage of the fact that E.

coli O157:H7 has limited growth above 44.5 C as this is distinguishable from most other E. coli.

Pathogenesis

The primary virulence factor associated with E. coli O157:H7 involve the organism's attachment to host intestinal lining and the subsequent production of cytotoxins (Karmali, 1989). The attachment of E. coli O157:H7 to intestinal microvilli is a key step in infection, as it allows the organism to colonize and resist peristaltic removal from the intestinal mucosa. It is suspected that electrolyte transport and carbohydrate absorption is inhibited as gastrointestinal tract colonization increases (Tzipori, 1989). The junction also allows for localized contact among toxins generated by the organism and the target intestinal epithelial cells. Histopathologic evidence shows that attaching and effacing lesions are present on intestinal walls infected with the organism (Tzipori, 1989). The specific factors associated with attachment to the intestinal wall are unclear but are known to involve a 60-megadalton (MDa) plasmid that expresses for a fimbrial antigen and promotes adherence to the target cells (Levine et al., 1987). This *eaeA* gene on the plasmid is apparently critical but not exclusive in the role of attachment (Jerse et al., 1990; Donnenberg et al., 1993). Other unknown outer membrane constituents are believed to contribute to the adherence of the organism (Dytoc et al., 1993; Sherman et al., 1991).

In addition to gastrointestinal tract adherence, E. coli O157:H7 is known to produce toxins that are cytotoxic to african green monkey kidney cells or Vero cells. These toxins are designated as verotoxins (VT) because of their cytolytic affects on

the Vero cells. The verotoxins VT1 and VT2 produced by the organism are similar with respect to biological activity, but differ in their immunological characteristics as they are antigenically dissimilar (Jackson et al., 1987; Strockbine 1988). Also, these verotoxins, VT1 and VT2, are known to be immunologically indistinguishable and identical in biological activities with Shiga toxins produced by Shigella dysenteriea and therefore are also referred to as shigalike toxins I and II (SLTI and SLTII), respectively. Both toxin types consist of an active polypeptide subunit A of 35 kilodaltons (kDa) that is surrounded by multiple copies of binding polypeptide B (10.7 kDa)(O'Brien and Holmes, 1987). The eukaryotic target binding site for both toxins is a globotriosyl ceramide consisting of a galactose α-(1-4)-galactose ceramide (Gb3) that is abundant in the cortex of the kidney and is present in the primary human endothelial cell cultures (Jacewicz et al., 1986; Lingwood et al., 1987; Wadell et al., 1988). Variants of these toxins have been isolated from certain strains of E. coli O157:H7, including SLT Iiv, which show good homology with SLT II but do not bind to the Gb3 receptor (Gyles et al., 1988; Weinstein et al., 1989). These toxins are all known to cause colonic injury, observed as eroded, ulcerated, and necrotized areas in gnotobiotic pigs infected with strains of E. coli O157:H7 (Weinstein et al., 1988; Francis et al., 1989).

Clinical Features

E. coli O157:H7 infection may go unreported especially if the individual is asymptomatic or experiences self-limiting nonbloody diarrhea, as these individuals do not typically undergo further complications. Most reported cases have been clinically diagnosed as hemorrhagic colitis observed as bloody diarrhea. E. coli O157:H7

infections may also exhibit more life-threatening diseases, including hemolytic-uremic syndrome (HUS) and thrombotic thrombocytopenic purapura (TTP).

Approximately 90% of E. coli O157:H7 infections lead to a mostly self-limiting condition known as hemorrhagic colitis (Tarr, 1995). Early stages of hemorrhagic colitis are usually difficult to diagnose as the initial symptoms may involve nonbloody diarrhea, a common symptom of most enteric diseases. Small bowel movement observed as nonbloody watery diarrhea is usually prevalent for up to 5 days of infection, followed by colitis onset, observed as bloody diarrhea (Ostroff et al., 1989a; Carter et al., 1987). An estimated 50% of all infected individuals suffer from vomiting with approximately a third developing a fever (Ostroff et al., 1989a). The manifestation of fever at such low frequencies allows physicians to differentiate E. coli O157:H7 infection from Shigella infections, typically associated with high fever.

HUS may develop in approximately 10% of all E. coli O157:H7 infected individuals (Tarr, 1995). HUS, described as a syndrome of microangiopathic hemolytic anemia, thromboctyopenia, and acute renal failure, is considered an extremely serious condition as it may lead to severe sequelae and in some cases death. Specifically, this condition most commonly affects children between 1 and 4 years old and may persist to severe renal or neurological disease. Approximately, 12% of children diagnosed with HUS show chronic renal impairment, neurologic damage, or hypertension (Siegler et al., 1994). Because shedding of E. coli O157:H7 cells occurs only 6 days after the onset of diarrhea, signs of E. coli O157:H7 cells after the development of HUS is improbable, as symptoms can range from several

days to weeks (Riley et al., 1983; Ashkenazi et al., 1988). Recent studies have shown that certain strains of E. coli O157:H7 are more likely to contribute to HUS. In particular, strains producing SLT II have lead to more cases of HUS than have strains generating SLT I or both SLT I and SLT II (Ostroff et al., 1989a; Thomas et al., 1991; Scotland et al., 1987).

Also, an extremely serious condition leading to sequelae, TTP is considered pathologically similar to HUS. Both HUS and TTP exhibit a form of microangiopathic hemolytic anemia while TTP demonstrates, profound thrombocytopenia, fluctuating neurological signs, high fever, and azotemia in elderly patients. The two conditions may be distinguished symptomatically as TTP shows a higher incidence in adults, and shows a lower prevalence of renal disorders with a higher frequency of neurological damage (Griffin et al., 1988).

Epidemiology

Since the E. coli O157:H7 outbreak of 1982, several efforts have been made to examine the epidemiology of the organism. Recent findings involve worldwide retrospective and prospective studies. So far, trends have been observed with respect to incidence, seasonal factors, age factors, and vehicles of transmission.

Incidence of E. coli O157:H7 has been discovered in several parts of the world but has shown higher prevalence in North America. Cases involving the organism have been observed in Japan and the United Kingdom but are considered very rare (Griffin et al, 1988; Smith et al., 1987; Cryan, 1990). A prospective study conducted by the CDC suggested that E. coli O157:H7 mediated hemorrhagic colitis occurred sporadically all throughout the United States (Remis et al., 1984). E. coli

O157:H7 infections rank third behind Salmonella and Campylobacter as the most common enteropathogen in the United States and Canada (Ostroff et al., 1989b). A Seattle based study suggested that an estimated 20,000 infections occur per year in the United States (MacDonald et al., 1988). Because incidents of E. coli O157:H7 infections are increasing each year, it is extremely difficult to conduct population-based studies with respect to sporadic and outbreak cases.

Seasonal factors are believed to contribute to these incidents of E. coli O157:H7 infection. A prospective studies have shown that incidence increased 60% over the warmer months between June and September (Ostroff et al., 1989b). Possible reasons for such high incidence over the months include greater colonization in animals and food items that serve as vehicles for transmission. Food items such as ground beef, important in the transmission of the organism, are consumed in greater quantities over the summer months. Presumably, a greater quantity of undercooked meat could result in such infections (Griffin and Tauxe, 1991).

Illnesses from infection by E. coli O157:H7 are also known to be influenced by age factors. Individuals who are very young or very old have a greater risk of infection (Spika et al., 1986; Griffin, 1995; Pai et al., 1987). Outbreaks have occurred in nursing homes where the highest incidence has been observed in the very old, while the younger staff members were not affected. Also, the youngest children in day-care centers experience the highest number of attacks. A study of an outbreak in a day-care center showed that children below the age of four showed a 4-fold higher attack rate than did older children (Spika et al., 1986).

Associated Sources

Animal sources

As previously described, occurrences of E. coli O157:H7 infections have been linked with food and nonfood vehicles of transmission. Improperly cooked ground beef has been implicated in several of these infections, according to studies on recent outbreaks (Griffin et al., 1998; Ryan et al., 1986). The largest outbreak in North America occurred in 1993, involving a fast-food chain in four western states. In this outbreak, over 700 people died and 195 people were hospitalized. Most of these victims were children of which 55 suffered from HUS while four died (Bell et al., 1994). Also from a bovine source, dairy products such as unpasteurized milk and pasteurized yogurt have been observed to be contaminated with E. coli O157:H7 (Morgan et al., 1993). The organism also has been isolated from foods of other animal sources such as seafood, pork, lamb and poultry products (Doyle and Schoeni, 1994; Samadpour et al., 1994).

Agricultural produce sources

In addition to animal vehicles of transmission, various agricultural products have been associated with E. coli O157:H7 mediated illnesses. Fruit and vegetable produce, including fresh pressed apple cider (discussed in the following fresh juice section), sprouts, lettuce, and potatoes have served as vehicles for E. coli O157:H7 associated outbreaks. Evidence indicates that E. coli O157:H7 contaminated produce has been associated with animal droppings that contain the pathogen. Ruminant animals, including cattle and deer, are suspected to defecate in crop fields. In the case of apple harvesters, the fruit are often picked from the ground rather than the tree,

increasing the chance that the apple may be contaminated with E. coli O157:H7 (Besser et al., 1993).

Nonfood sources

Outbreaks also have resulted from waterborne transmission, mostly involving crop irrigation. Water served as the vehicle in an outbreak of E. coli O157:H7 from drinking nonchlorinated water and from swimming in a lake containing significant amounts of the organism (Swerdlow et al., 1992; Keene et al., 1994). More recently, studies suggest that E. coli O157:H7 infections can spread from person to person. This evidence was revealed from epidemiological surveys of outbreaks, observing that secondary infections developed in nursing homes, day care centers and among family members. Studies of a large outbreak in 1993 showed that approximately 11% of cases resulted from person-to-person transmission. This evidence complicates further investigations into the control of the organism, as health officials may have to locate persons who have contacted infected individuals to prevent further spread (Ostroff et al., 1989b).

Adaptive Resistance to Environmental Stress

Acid Tolerance

Bacteria belonging to the family enterobacteriaceae are considered neutraphilic as they grow optimally at pH between 6.8 to 7.2. However, some of these bacteria are able to survive and in some cases grow over a wide pH range. Of particular concern to the food industry are bacterial pathogens that are able to survive low-pH. These acid-tolerant microorganisms are more difficult to control in the food-processing environment, as several antimicrobial agents used to clean processing

equipment are acidic in nature. Also, these acid tolerant microorganisms are able to survive low pH food products including fresh juices, once thought to be safe without the application of extra processing measures such as pasteurization.

Low pH conditions are typically lethal to neutrophiles, as membranes, enzymes and DNA become degraded by protons, with damage to the DNA being most critical for the killing effect according to Sinha (1986). To reduce injury at less than neutral pH, neutrophiles are known to induce cell homeostasis responses. Bacterial regulation of cell homeostasis is not fully understood, and is believed to involve several different mechanisms. Bacteria achieve homeostasis by regulating the flow of protons in and out of the cell to maintain a constant internal pH of approximately 7.6. If intracellular pH becomes too low, protons are pumped from the cytoplasm through a membrane-bound proton pump to form a trans-membrane electrochemical gradient (Mitchell, 1973). The gradient provides a barrier against protons that would ordinarily penetrate the cell membrane. If extracellular proton concentrations are too high and exceed the trans-membrane threshold level, the gradient becomes overwhelmed, which permits protons to pass through to the cytoplasm.

Under special circumstances, bacteria are able survive high acid situations by initiating mechanisms that allow the organisms to adapt to the low pH. Salmonella spp. and E. coli have survived extremely low pH, after being pre-exposed to moderately low pH of 5 and 6 (Foster, 1991). Initially characterized for Salmonella typhimurium, the adaptive response is referred to as acidification tolerance response (ATR). This response by Salmonella spp. and E. coli is a result of two protein

synthesis events. The first event occurs during the adapted phase (preshock) as cells are incubated at moderately low pH (pH 5 to 6). The bacteria proliferate after undergoing a slight lag period resulting from the acid exposure. After several generations of growth, the bacteria develop resistance factors, by synthesizing acid shock proteins (ASPs) that allow the cell to resist extremely low pH (pH 3 to 4) (Foster and Hall, 1990). Lee et al. (1994) showed that a "major molecular realignment" occurs at during adaptation at pH 5-6 and that 40 proteins are induced in Salmonella typhimurium. Foster (1991), found pre-shocked Salmonella spp. cells that are adapted to a pH of 5.8 express 12 proteins and repress 6 proteins after comparing the adapted cells to cells that are growing at neutral pH. Hickey and Hirshfield (1990) observed that a 2- to 14-fold increase in production of 13 E. coli and 19 S. typhimurium was induced after shifting the cells from pH 7.0 to pH 5.0 when compared to controls. When the bacteria are subjected to an extremely low pH, or acid shocked, a separate set of proteins, referred to as acid shock proteins (ASP) may be synthesized that protect the cell from injury or aid in repair (Raja et al., 1991). Acid shock response (ASR), as a result of shifting S. typhimurium from moderately low pH to extremely low pH, induces the production of 37 proteins and the repression of 15 proteins when comparing to a culture grown at neutral pH. The function of this group of acid shock proteins is unclear as inhibiting protein synthesis at this stage did not have an effect on acid tolerance (Foster, 1991). A direct shift from neutral pH (6.9) to low pH (4.3) in E. coli induces the formation of 16 polypeptides including 4 heat-shock proteins, 3 stress-related proteins, and 7 acid-shock proteins (Heyde and Portelier, 1990).

Temperature effects on acid resistance have been characterized in several studies. Brudzinski and Harrison (1997) found that E. coli O157:H7 cells subjected to elevated temperatures show greater resistance to acid. They observed that stationary-phase E. coli O157 that are statically incubated at 32°C showed more resistance to acid than did cultures incubated at 25°C. Temperature effects on acid resistance also were studied by comparing survival of E. coli O157:H7 isolated from beef, salami, and apple cider in the presence of lactic acid. These strains were all observed to be more resistant to lactic acid levels of 0.1%, 0.25%, and 0.5% when cultured at 37°C over 10°C (Semanchek and Golden, 1998). Wang and Doyle (1998) showed that certain strains of E. coli O157:H7 subjected to heat shock of 10 min. at 48°C showed 10 to 100 times greater survival than did cells that were left untreated. This study showed that no significant differences in acid resistance between samples that were heat shocked and samples that were acid treated.

The ATR and ASR have been characterized further according to nutrient conditions and growth phase. This adaptive response also been was more pronounced with starved, stationary-phase cells. One reason for this is that stationary-phase cells in nutrient-deprived conditions have a higher proportion of completed chromosomes and the presence of replication forks is believed to increase susceptibility to inhibition by external factors such as pH. Studies also distinguished stationary phase cells from log phase cells with respect to acid tolerance responses. Lee et al. (1994) found that ATR by stationary phase S. typhimurium was more resistant to acid than log phase cells, despite the synthesis of fewer proteins. Arnold and Kaspar (1995) agreed with this when comparing mid log and late log phase cells to late stationary phase E. coli

O157:H7 cells. This study also confirmed that starved log phase cells showed higher acid resistance than log-phase cells in nutritive conditions. A study by Jordan et al, (1999) agreed with this, as it was determined that stationary phase E. coli O157:H7 underwent cell envelope modifications with respect to protein composition after adapting to low pH. These modifications were suggested to have an influence on proton flux into the cell but the authors could not determine if the mechanism was passive or active. The higher acid tolerance of nutrient deprived and stationary phase cells are due to activity of a general stress response system (Lin et al., 1995). This response system, also affected by elevated temperatures, is a function of the RpoS sigma factor, known to initiate the generation of protective proteins and promote cellular modifications associated with the acid tolerance and acid shock response.

Despite several efforts to clarify the role of rpoS, in acid resistance is still not fully understood but strongly believed to affect vital cellular components including the outer membrane. Cheville et al., (1996) found that starvation induced acid tolerance was not observed with RpoS negative mutants, which showed much less resistance to acid, heat and salt factors than those cells that maintained the regulatory factor. RpoS regulatory factors are suggested to influence the synthesis of cyclopropane fatty acid (CFA) that increase E. coli acid resistance. The formation of CFA is a postsynthetic modification of the lipid bilayer as bacteria enter the stationary phase (Chang and Cronan, 1999). Wang and Doyle (1998) suggested that the synthesis of two outer membrane proteins, that may be initiated by RpoS during and after heat shock, was important to protecting the cells from acid..

Lin et al. (1996) conducted extensive studies on rpos role and function. They described an rpoS influenced arginine and glutamate -dependent system protective system. In their study they examined acid-adapted E.coli O157:H7 in the presence of benzoic and other acids (acetic, propionic, and butyric acids) mixed with fatty acids at levels that simulate conditions in the intestines. It was determined that the arginine-dependent system allowed for better protection of E. coli O157:H7 than other commensal strains when subjected to a pH 2 solution. The glutamate-dependent system functioned for all strains studied and showed greater effects than the arginine-dependent system. The authors found that E.coli O157:H7 adaptive protection is maintained for extended periods under refrigeration temperatures. According to Lin et al. (1996) proteins synthesized by E. coli O157:H7 may protect the cell through decarboxylation of the amino acids by protons that enter the cell, thereby maintaining consistent intracellular pH.

Several survival studies support the idea that rpoS plays a significant role in acid resistance. However, some studies have suggested that alternate mechanisms are involved as rpoS negative mutants have are able to withstand low pH (Lee et al., 1994; Small et al., 1994). Foster and Hall (1990) observed phosphate regulation factor (phoP) negative Salmonella typhimurium was 1,000 fold more acid sensitive than wild type cells. In addition, these researchers suggested that magnesium dependent translocating ATPase also plays an important role in acid tolerance as organisms missing these determinants are easily inactivated in acidic environments.

Cross Protection

In addition to exhibiting tolerances to extremely low pH, stress adapted bacteria have shown greater survival over nonadapted bacteria when subjected to other environmental factors including heat, sodium chloride and osmolarity challenges. Although the phenomenon is not fully understood, increased cellular survival under antimicrobial influences is suspected to also be a result of stress related and general stationary phase related mechanisms. Proteins sythesized during a stress adaptation period are believed to increase the stability of the bacteria under adverse conditions. This form of resistance, referred to as cross protection, has been observed with several Gram-negative cells, and has allowed for cellular resistance towards a variety of stress factors. Cross protection was observed in a study by Leyer and Johnson (1992) showing that adapted S. tymphimurium survived better than nonadapted organisms during cheese manufacture and curing of cheeses. This finding was based on comparing acid-adapted to nonadapted cells after exposure to antimicrobial factors including H_2O_2, bacteriocins, salt, low water activity, lysozyme, lactoperoxidase, and bacteriocidal lipids. A follow up study, conducted by Leyer and Johnson (1993) revealed that an adapted S. typhimurium showed greater thermal resistance at 50°C than nonadapted cells as a 10-fold difference was observed after 20 min. of exposure. This 10-fold difference was also seen at time temperature combinations of 55°C for 10 min. and 57.5°C for 2.5 min. The study indicated that cross protection also existed against salt, activated lactoperoxidase system as well as surface agents including crystal violet and polymyxin B. Conversely, Ganzle et al. (1999) found that –LPS mutant E. coli and Salmonella showed limited protection

against the bacteriocins, nisin and curvicin A, after exposure to an acidic environment. However, difference may be due to variating acid adaptive methods. Jenkins et al. (1990) discovered that nutrient deprived, stationary phase E. coli exhibited cross protection towards NaCl concentrations of 2.2 to 2.4 M.

Several cross protection studies on E. coli O157:H7 have focused on comparing stress resistances of acid-adapted cells to acid shocked cells as two different groups of proteins have been associated with each. Acid-adapted and shocked E. coli O157:H7 cells were compared by Garren et al. (1998) after introduction to elevated concentrations of sodium chloride (5%, 10%, or 15%) and sodium lactate (10%, 20%, or 30%). Acid-adapted E. coli O157:H7, with exception to one strain, showed greatest survival under all conditions. A similar study by Rowe and Kirk (1999) involved acid shocking E. coli O157:H7 cells at pH 3.5, 4.0, 4.5, and 5.0 and subjecting them to 20% w/v NaCl and heat at 56°C. It was determined that certain strains showed significant resistance to heat or salt when prestressed at pH 4.0.

E. coli O157:H7 cross protection studies have also been performed using a variety of matrices. A study by Ryu and Beauchat (1995) compared acid-adapted and shocked E. coli O157:H7, to evaluate cross protection against heat in fruit juices as adapted organisms exhibited greater resistance than those that were shocked. A similar study by Ryu and Beuchat (1999) focused on heat tolerance of acid-adapted and acid shocked E. coli O157:H7 in TSB supplemented with glucose. Again, it was concluded that acid-adapted cells generally allowed for significantly ($\alpha = 0.05$) greater resistance to thermal treatments of 52°C, 54°C and 56°C. D-values at 52°C

and 54°C were higher for adapted and shocked cells than nonadapted cells in the two brands of orange juice and apple juice tested. Ryu and Beuchat (1999) found that acid shocked and acid-adapted E. coli O157:H7 showed no significant cross protection against dehydration (a_w = 3.4±0.06, 6.8±0.01) and high salt (0.5, 3, 20%) conditions in beef powder. The effects of glucose supplementation on cross protection of acid-adapted E. coli O157:H7 cells was studied by Buchanan and Edelson (1996). It was determined that cells grown on acidified, glucose (1%) supplemented TSB showed significantly higher resistance to heat (56 – 62°C) than TSB without glucose for chicken broth, UHT milk but not apple juice.

The exact mechanisms behind cross protection are unclear, but may be in large part due to rpoS regulation as discussed in the acid-adapted section. This rpoS response has been studied to be induced as early as 15 min. after exposure to stress. Several studies have suggested that other factors are involved in cross protection as rpoS negative mutants show protection. One possible mechanism involves Leyer and Johnson (1993) finding that cell hydrophobicity and outer membrane proteins (ompC) increase in Salmonella typhimurium after osmotic shock. Sato et al. (2000) also determined that outer membrane proteins, ompC and ompF are generated by E. coli in low pH conditions. These proteins allow for porin formation which regulate the passage of compounds in and out of the cell. These protection factors may collectively contribute to survival of stress habituated cells.

Apple Juices and Ciders

Acid foods, including fruit juices and ciders, were once considered relatively safe as pathogenic bacteria were not believed to survive in low pH environments. Fruit juices and ciders, consist of weak acids allowing for a final pH of less than 4.6. Apple ciders and juices are generally unfavorable for bacteria survival, as they typically have a pH of 4.0, due to malic and lactic acids in the product. Other properties associated with apple juice and cider, including sugar content (°Brix) and presence of pulp are also known to play a role in microbial survival.

The fresh juice industry has suffered from a recent series of outbreaks associated with fresh juice products. Several of these outbreaks have resulted from the consumption of processed apple beverages such as juices and cider. In a 1974 outbreak involving apple cider, 296 people became ill from the ingestion of Salmonella typhimurium (CDC, 1976). In 1980, E. coli O157:H7 was suggested to be the causative agent in an outbreak in Canada as 14 children were diagnosed with HUS after they consumed apple juice (Steele et al., 1982). In 1991 E. coli O157:H7 was implicated in an apple cider outbreak in Fall river, Massachussetts where 18 individuals became ill. Patients infected with E. coli O157:H7 were diagnosed with non-bloody diarrhea, bloody diarrhea, abdominal pain, acute renal failure, or HUS (Besser et al., 1993). An investigation of a 1996 multistate outbreak (British columbia, California, Colorado, Washington) indicated 45 illnesses occurred, resulting from the consumption of apple juice contaminated with E. coli O157:H7. Patients infected with the organisms ranged from 1 to 41 years old with the median age of 5 years. Reports have suggested that contamination of these apple based

products may have resulted from processing "dropped" apples that were exposed to deer feces (CDC, 1997).

The increased frequency of fresh juice and cider associated outbreaks in the past decade has influenced the Food and Drug Administration to consider intensifying regulatory activity on the industry. The FDA currently supports three measures that will affect the fresh juice industry including a new label requirement, safety control program, and microbial inactivation specifications. The label requirement mandates the presence of a warning statement on the label of unpasteurized fresh fruit juice products informing the consumer of the risks of drinking unpasteurized products. The warning statement should read: "WARNING: This product has not been pasteurized and, therefore, may contain harmful bacteria which can cause serious illness in children, the elderly, and persons with weakened immune systems". This regulation is currently in final rule status and currently affects the fresh juice industry (USFDA, 1998a). The proposed safety control program, is designated "Hazard Analysis at Critical Control Points" (HACCP) is designed to reduce potential hazards in processing as well as to force processors to keep updated records of events (USFDA, 1998b). The proposed decrease in product microbial load enforces a five-log reduction in microbial activity from the point of harvesting and prior to distribution. This can be achieved by implementing intervention strategies that are approved by the FDA (USFDA, 1998b). Due to potential regulatory activity by the FDA, scientists are studying the effectiveness of various approved intervention practices including thermal, benzoate/sorbate, and freeze-thaw treatments, on reducing microbial counts in fresh juice products.

Pasteurized fruit juices are typically heated at 68°C for 20 to 30 min. or 82°C for 20 to 30 seconds. However, this treatment compromises quality characteristics, including flavor, associated with fresh juices. Therefore, studies have been conducted to observe the effects of applying lower levels of heat to examine efficiency in reducing microbial load. A study by Splittstoeser et al., (1995) involved applying thermal treatments on apple juice samples containing E. coli O157:H7. D-values for 52°C, 55°C, and 58°C were determined to be 12, 5.0, and 1.0 min. respectively. According to the study, °Brix (11.8, 14.2, and 16.5), % L-malic acid (0.2, 0.4, and 0.8) and pH (3.6, 4.0, and 4.4) associated with the apple juice samples had no effect on the D-values for the samples. Ingham and Uljas (1998) stored apple juice and cider at room temperature of 21°C prior to subjecting the juice to more elevated temperature of 61°C as a possible means of reducing E. coli O157:H7 counts. It was found that short term room temperature storage (≤ 6 hours) may increase inactivation of E. coli in apple cider and apple juice (pH 3.4) after subjecting samples to 61°C. This study also indicated that E. coli O157:H7 heat resistance is strain specific. Semanchek and Golden (1998) compared three strains of E. coli O157:H7 isolated from ground beef, apple cider, and salami to determine the effects of growth temperatures of 10°C and 37°C on thermal inactivation at temperatures of 52°C, 54°C, and 56°C in 0.1% peptone water. In general, the salami strain had the highest D-values while the beef strain had the lowest D-values.

In attempts to avoid applying harsh heat treatments on apple juice and apple cider, some processors use the preservatives benzoate and sorbate. Benzoate mostly targets yeasts and molds while sorbate inhibits some bacteria in addition to yeast and

molds. A study by Zhao et al. (1993) indicated that the FDA approved levels of 0.1 % potassium sorbate and 0.1% sodium benzoate combined effectively decreases the E. coli O157:H7 counts in apple cider held at 8°C or 25°C. This was observed as the E. coli O157:H7 could not be recovered from most treated samples after 3 days while the organism survived for up to 28 days in control samples without preservatives. Conversely, Miller and Kaspar (1994) reported that potassium sorbate and sodium benzoate showed minimal effects against E. coli O157:H7 in apple cider with 95% survival after 21 days when held at 4°C and 21°C. The authors suggest that the difference in findings may be due to strain variation and factors inherent to the cider. Nonetheless, these conflicting reports indicate a further need to elucidate the synergistic effects of benzoate/sorbate treatment.

Some apple juice and apple cider processors freeze their products during storage and distribution to preserve the product. Recently, studies have indicated that the freezing may significantly inactivate microorganisms. According to Semanchek and Golden (1998), E. coli O157:H7 strains isolated from ground beef, or salami, apple cider that were cultured at 10°C and 37°C and frozen for 7 months at –20°C showed a 4- to 6-log CFU/mL decrease in survival in 0.1 % peptone water. It was determined in this study that the cider strain showed higher D-values than the beef and salami strains. Uljas and Ingham (1998) performed a study on apple cider samples inoculated with E. coli O157:H7 stored at 4°C, 25°C, or 35°C prior to freezing at –20°C for 48 hours with subsequent thawing at 4°C for 4 hours. This study showed that apple cider with a pH of 4.1 showed a 4-log decrease in microbial activity after storage at 35°C for up to 6 hours prior to the freeze-thaw cycle.

Samples that were adjusted to a pH of 3.3 showed no survival after the freeze-thaw cycle. These studies indicate that the effects of freezing are significant in reducing microbial load.

Sublethal Injury

Strong evidence from several survival studies has suggested that actual cell counts after stress treatment are greater than can be observed using conventional culturing methods. The bacteria that survive the treatment, but have temporarily lost the ability to multiply on some media, are considered to be in the sublethal injury state. Sublethally injured cells are of public health significance as their incapacitated condition is transient and they may still maintain the ability to infect a host. Therefore, recent studies have focused on further characterizing the sublethally injured state along with refining methods for recovery and detection.

Sublethally injured cells are known to suffer and recover from 'hits' at various critical intracellular and extracellular target sites. These targets include the cell wall, cytoplasmic membrane, and nucleic acids depending on the source of stress. Cells that suffer from nonlethal hits typically experience extended lag phases in nutritive media. Recovery from injury is not fully understood, but is believed to involve repair mechanisms specific for the impaired components. Optimal conditions for recovery such as nutritional requirements, temperature effects, and oxidative considerations have been extensively characterized. These conditions have been utilized to improve detection of sublethally injured cells.

Traditional Enumeration Techniques

Standard techniques designed to enumerate sublethal injury cells, involves distributing stressed cells to optimum medium (e.g. nutritive), to determine total viable counts, and restrictive medium (e.g. containing salts), that allows for growth of only the uninjured cells. Sublethal injury can be measured as the difference of counts between the two media. A resuscitation step may be required to allow injured cells to recuperate sufficiently enough to colonize the optimum medium. The basis for this type of analysis has also been successfully used with most probable number procedures. Limitations to these type of methods includes the length of time and lack of reliability as sublethally injured cells that are non-culturable will not be detected (Wilshaw et al., 2000).

Flow Cytometry

More recent techniques for enumeration of sublethally injured cells, involves the use of flow cytometric methods. The technique of flow cytometry forces a suspended bacteria to pass through a well defined flow channel one by one for measurement by a photosensor. The photosensor has potential of measuring total nucleic acid content, surface antigens rRNA and viability depending on the fluorescent labels that are typically used in combination with the technique. Uses of flow cytometry to assess viability has involved staining the organisms with flourogenic dyes or introducing them to chromogens that are specific for cellular components or properties associated with an active cell. Recently, chromogens, such as carboxy-flouorescein-diacetate (CFDA) and biscarboxyethyl-carboxy-fluorescein (BCECF) that are specific for esterase activity in combination with flow cytometry

have proved to be very valuable in enumerating sublethally injured cells. This system has the potential of detecting several thousand viable cells per second. However, viability counts may be inaccurate, as it is possible for dye leakage to result from cell extrusion of the fluoroscein product (Ueckert et al., 1995; Seo et al., 1998)

Bioluminescence

Luminous bacteria are can be found in terrestrial, freshwater and marine environments. Determinants coding for bioluminescence production have been isolated from marine halophiles including Vibrio fischeri, Vibrio harveyi, Photobacterium leignathi, P. phosphoreum, and Xenorhabdus luminescens. These genes have been used as phenotypic determinants for reporting gene expression and cell viability (Meighen, 1993). Bioluminescence is generated naturally through the expression of an operon that may be species specific but primarily involves the genes *luxCDABE*. A heterodimeric enzyme, luciferase, is encoded for by the *luxAB* genes while the *luxCDE* determinants allow for the production of the long chain aliphatic aldehyde, tetradecanal (Stewart and Williams, 1992). Bioluminescence is observed when luciferase catalyzes the endogenous oxidation of an aliphatic aldehyde and the reduced form of flavin mononucleotide ($FMNH_2$) by molecular oxygen thus emitting light photons. Since $FMNH_2$ is a product of electron transport, only viable cells containing the lux operon express bioluminescence.

Bioluminescent marked cells may be derived by transforming the host organism with lux CDABE or *luxAB*. Organisms transformed using *luxAB* must be provided with an exogeneous solution of a long chain aliphatic aldehyde, such as dodocenal or tetradecanal, that serve as a autoinducer, essential for the

bioluminsecence reaction to occur. Since transformation using *luxCDABE* allows the recombinant cell to produce its own source of the tetradecanal, the bioluminescence reaction is not dependent on the addition of the autoinducer. Bioluminescence is quantitated by measuring photons per second expressed as relative light units (RLU). RLU is measured using a luminometer device or scintillation counter that is sensitive to the photons (~450 nm) expressed by the transformants. Images of bioluminescent cells can be observed using charge-coupled device (CCD) cameras. This allows for spatial analysis as images of cell distribution and location can be provided. The *lux* genes are considered valuable markers as they allow for phenotypic expression without destroying the cell. Also, bacterial bioluminescence is highly sensitive and permits real time analysis of cells expressing luciferase. The techniques involved in quantitating the marked bacteria are relatively rapid, as methods typically do not involve dilutions.

Several studies have compared conventional plating methods with the *lux* based system to study sublethal injury and survival of bacterial cells under a variety of conditions. Ellison and Stewart (1991) found that viable plate count and RLU values showed good correlation when using a lux AB marked S. typhimurium frozen at -20°C with subsequent thawing. Duffy et al. (1995) also compared bioluminescence with plate count methods in a thermal inactivation study using a plasmid-mediated *luxAB* marked S. typhimurium. It was found in this study that severely heat treated cells could not be observed using the plate count method while RLU values could still be recorded. The investigators suggested that

bioluminescence was a more practical tool to quantitate cell numbers at lower survival levels than conventional plate count methods.

Bioluminescence has also proven to be a valuable tool when used in food systems. In a study by Chen and Griffiths (1996), a S. enteritidis was transformed using a transposon carrying the *luxAB* genes and inoculated in homogenized chicken meat, whole liquid eggs, and fluid milk. It was determined that the luminescence was maintained by the organism during acid and heat treatments, however RLU's were slightly lower for food samples than pure culture broth samples. This phenomenon was attributed to the "quenching effect" that the food samples may have had on the light produced by the bacteria. A recombinant *luxCDABE* Salmonella hadar was used to measure the effect of acid and temperature in lactic acid–treated turkey carcasses. The investigators found that the recombinant strain maintained wild type morphological, growth, biochemical characteristics after transformation with the cassette. Also, it was determined that the transformant maintained the *lux* plasmid at a temperature of 40°C and pH of 4.02 after 10 and 60 min. of exposure, respectively (Bautista et al., 1998).

The use of *lux* recombinant E. coli O157:H7 has also been valuable to study their control in foods. Ramsaran et al. (1998) found that the use of a bioluminescent marked E. coli O157:H7 to measure their activity during the manufacture of Feta and Camembert cheeses was very reliable. A bioluminescent marked E. coli O157:H7 was also used in a study by Hudson et al., to measure survival in yogurt at different pH (4.17, 4.39, and 4.47) and storage temperatures (4°C and 10°C). The recombinant organisms was also used to monitor the survival during the manufacture, curing and

storage of Cottage, Colby, Romano, and Feta cheeses according to pH, starter culture presence and temperature. The method proved to be more rapid and practical than plating methods.

A *lux* recombinant E. coli O157:H7 was also used to quantitate and study spatial distribution of the organism on beef carcasses. Good correlation ($R^2 = 0.97$) was achieved when comparing RLU's to plate counts. Images of the location and distribution of the recombinant organism on beef carcasses was also valuable to determine the tissue types that the organism has greatest affinity for. These studies found that bioluminescence proved to be very promising in measuring sublethal survival of bacteria in food after treatments (Siragusa et al., 1999).

MATERIALS AND METHODS

Bacterial Strains

The bacterial strains used in this study include three E. coli O157:H7 strains designated as 9124-1, mf6707.a, and mf1847 the strains are referred to as strain A, B, and C respectively in this study. A non-O157:H7 designated EcoR1 was used as a control. The O157:H7 strains are environmental isolates obtained from Dr. Keith R. Schneider at ABC Research Corporation, Inc. (Gainesville, FL) and the non-0157:H7 control was obtained from American Type Culture Collection. E. coli O157:H7 were confirmed according to Food and Drug Administration (FDA) approved procedures as described in the Bacteriological Analytical Manual. In addition, API20e biochemical analyses were performed after 24-hour incubation at 37°C on all organisms used in the study according to procedures illustrated in the "20E® analytical profile index" 9th edition for enterobacteriaceae and other gram-negative bacteria by analytab products (Sylsset, NY 11791).

All strains involved in the study were cultured overnight on Difco® Tryptic Soy Agar (TSA) or in Tryptic Soy Broth (TSB) at 35°C. Recombinant organisms derived in the study were cultured on TSA or TSB containing 100 μg of ampicillin (Sigma®; St. Louis, MO 63178) per liter. Cultures were stored at 4°C on TSA or TSB with or without ampicillin for up to 8 days before reculturing to allow for frequent

usage. Stock cultures were stored at –20°C in 15% glycerol-TSB with or without ampicillin media for up to 3 months before reculturing

Plasmid Maintenance

A solution of plasmid pT7-5, obtained from Dr. M. Griffiths's laboratory at University of Guelph (Guelph, Ontario Canada), and was derived at Dr. E. Meighen's laboratory at McGill University (Montreal, Quebec Canada). This plasmid is a 2.4 kb plasmid that features a *luxABCDE* sequence encoding luciferase and an aliphatic aldehyde. The plasmid also carries a sequence conferring ampicillin resistance. Supply of the plasmid was replenished by transforming 10^8 competent JM109 cells obtained from Promega® (Madison, WI 53711-5399) using pT7-5 plasmid according to Hanahan procedures. These cells were cultured on TSA or TSB containing 100μg/L ampicillin. Plasmid DNA was extracted from the competent cells using Promega's Wizard™ Plus miniprep kit according to the procedures described in the Wizard miniprep booklet. Plasmid solutions were resuspended in deionized H_2O and stored at –20°C.

Apple Cider Characteristics

The apple cider used in this study was a gift from Dr. Murat Balabans laboratory at the University of Florida, who received the apple cider from Dr. John Roberts from Cornell University (Ithaca, NY 14853-2801). Dr John Roberts explained the preparation of apple cider samples as follows: "Two bins of apples (1) Gala, (1) Liberty were purchased from the program of Susan Brown of Cornell. Apples were washed using the bin dumper, conveyor, and water brush in the Raw Products facility, placed into plastic bushel crates, and stored overnight at 32°F (0°C).

The next morning apples were moved to the pilot plant for pressing according to the following procedure: Alternate bushels of Gala and Liberty were passed through a hammermill with speed on high, a 3/4 inch (1.91 cm) screen and the blunt side of the hammers forward. The resulting mash was then layered into a plate and frame hydraulic press in approximately 18 inch (45.72 cm) square by 3 to 4 inch (7.62 to 10.16 cm) thick layers on conventional press cloth covered with a single layer of cheese cloth. After sufficient layers were made the "stack" was partially pressed. This allows for the addition of several more layers. The entire stack was then pressed to about 2000 lbs (907.18 kg). pressure until juice stops flowing. This process was repeated until approximately 120 gal. (454.24 L) of cider was obtained. Cider was stored at 32°F (0°C) overnight before filling. Filling was accomplished by bucketing the stirred cider by hand into a 20 gal. (75.71 L) stainless kettle fitted with a butterfly valve. Bucketing was done alternately from the several containers of juice to further insure uniform blending of the two varieties. One-half gallon (1.89 L) containers were then filled and capped by hand and placed at -40°F (-40°C) for freezing until shipping. Cider was shipped at –40°F (-40°C) and received at UF on October 28, 1999 and for frozen storage (-20°C)."

The half- gallon (1.89 L) jugs were thawed at 4°C for 24 hours and kept at 4°C prior to usage. The samples were then filtered through a 0.45 [m pore-size Nalgene® (Rochester, NY 14602-0365) filter to remove pulp, and handled aseptically when distributing samples for further analyses.

Apple cider was analyzed for microbiological and chemical properties. Microbiological analyses were done to determine the amount of background flora

before and after filtration. One mL samples was diluted in 9 mL Butterfields phosphate buffer to 10^{-3} dilution, with 0.1 mL from each dilution spread on orange serum agar (Difco; (BD Biosciences; Sparks, MD 21152). Chemical analyses were done by measuring acidity and soluble solids.

Electrotransformation

All bacterial organisms used in this study were cultured in TSB at 35°C until the optical density at 600 nm (OD_{600}) reached 0.8, determined using a Beckman DU-600 spectrophotometer (Beckman-Coulter; Fullerton, CA 92843-3100). The cells were then prepared for electroporation using a procedure illustrated by Calvin and Hanawalt (1988) with minor deviations. Overnight cultures grown in TSB (10 mL), were chilled on ice for 2 hours and harvested in a Dupont Sorvall RC-5B superspeed refrigerated centrifuge at 4000 *g* for 10 min. at 4°C. These cells were then washed once with 10 mL ice-cold , deionized water-10% (w/v) glycerol solution at 4000 *g* for 10 min. The resulting pellet was then resuspended in 100 µL deionized water-glycerol solution and distributed to BIORAD Gene Pulser/ E. coli Pulser (0.2 cm) cuvettes (Cambridge, MA 02139) that were chilled on ice for an hour.

A 10 µL pT7-5 solution at 100 µg/ L was then pipetted into chilled cuvettes containing cells and lightly shaken. The samples were then electroporated using a BIORAD® Gene Pulser II at a voltage of 2.4 kV and with resistance and capacitance settings at 200 Ohms and 25uF, respectively. After pulsing, cells were gently resuspended in 900 µL TSB and incubated at 35°C for 50 min. to allow for recovery of injured cells. The TSB is then spread on TSA-AMP and incubated overnight at 35°C. Colony formation on the antibiotic media indicated possible transformants.

To confirm that the plasmid DNA pT7-5 was inside the transformed cells, the plasmid DNA was extracted using the Promega Wizard™ (Madison, WI 53711-5399) miniprep system. The plasmid DNA was then digested using 1U of Hindi III endonuclease enzyme according to manufacturer's instructions. Standard gel electrophoresis techniques were performed on the digest using Tris-Borate-EDTA (pH 8.0) buffer at 60 volts. The size of the digested DNA was determined by comparing resulting band distances with sized bands from a Hindi III digested lambda DNA standard.

E. coli O157:H7 were also analyzed for physical or biochemical changes that may result from transformation with foreign DNA. Possible morphological differences were assessed by comparing wild type E. coli O157:H7 with recombinant E. coli O157 by Gram staining cells and viewing under a compound microscope at 100x magnification. Also, wild type and recombinant E. coli O157:H7 comparisons were made based on colony formation and serotype while performing the FDA approved BAM procedures. Possible differences in biochemical features between wild type and recombinant E. coli O157:H7 were compared using results from API20E strips.

Acid-adapted and Nonadapted E. coli Cell Preparation

The acid-adapted and nonadapted bacteria were prepared so that both would undergo an equal number of washings to compensate for possible decrease in cell counts resulting from the washes. These conditioning procedures were done simultaneously for both groups of cells resulting in comparable cell numbers between groups prior to each treatment. Bacteria used in this study were acid-adapted by

initial culturing in Vogel-Bonner minimal media or E-medium (pH 6.8), containing 1% glucose, followed by growth in moderately acidified media. Bacteria were subcultured in 100 mL of minimal media for 16 hours at 35°C to provide late stationary phase cells. An OD_{600} was measured (~0.70) and a 1 mL transfer was made from the E-medium culture to 100 mL E-medium at pH 6.8 or adjusted to pH 5.0 using 1 N HCl to provide nonadapted and acid-adapted cells, respectively. These solutions were incubated at 35°C for 22 hours or until OD_{600} measurement (~0.70) reached the same value as that obtained from culture grown in the pH 6.8 E-medium. A 1.3 mL aliquot from the pH 5.0 and pH 6.8 E-medium was transferred into 1.5 mL eppendorf tubes and washed in 1.3 mL Butterfields phosphate buffer (pH 6.8) using Brinkman® eppendorf model 5415 microcentrifuge (Brinkman Instruments, Inc.; Westbury, NY). The resulting pellet was ready for resuspension in the appropriate matrix for treatment.

Luminometry

A 1 mL sample of cellular solution was distributed into Biolumac™ cuvettes. The cuvettes were placed in a 30°C water bath for 10 min.. A 0.1 mL aliquot of 0.1% dodecanal solution (Sigma®, inc.) was pipetted into the cell suspension and shaken for 3 seconds. The cuvette was placed into a Biolumac Biocounter™ M2000 luminometer which was set to obtain triplicate readings every 10 seconds.

Linear Regression Analyses

Linear regression analyses were done to establish correlation between colony-forming units/ mL and relative light units. Good correlation (R^2>0.95) is important in predicting colony-forming unit values based on RLU measurements. Since

deviations may exist among factors (acid adaptation vs. non-adaptation; E medium vs. buffer vs. apple cider) correlation was evaluated for all six factor combinations. Linear regression analyses was done on survival data obtained by subculturing organisms in 100 mL E-medium and incubating for 16 hours at 35°C. A 0.1 mL aliquot from this culture was then cultured in 100 mL E-medium for 16 hours. A 1.3 mL (or 1.5 mL sample for cells to be treated by freeze-thaw) sample of the stationary phase cells were then harvested by microcentrifugation at 10,000 g for 3 min. and resuspended in 1.3 mL BPB, apple cider, or E-medium and incubated at 20°C for 9 hours. The cultures were washed and resuspended in 1.3 mL BPB and diluted 10^{-1} to 10^{-10} accordingly in BPB. Counts for each dilution were obtained by spread plate method on TSA-AMP and luminometry. Regression analyses were conducted on the data obtained and a linear regression model was developed from data plots.

Statistical Analyses

Statistical analyses, using T-test assuming unequal variances (Microsoft® Excel 97 SR-1), were applied to evaluate transformant stability, sublethal injury, and cross protection in this study. Transformant stability is described in the following section. Sublethal injury was assumed if RLU-derived count triplicate D-values obtained using RLU-derived counts were significantly higher ($\alpha = 0.05$) than triplicate D-values obtained using plate counts. Cross protection was observed if acid-adapted triplicate D-values were significantly higher than nonadapted triplicate D-values.

Transformant Stabilization

Verification of plasmid stability was assessed by statistically comparing (T-test assuming unequal variances) predicted counts before and after treatment using RLU-derived counts obtained from linear regression analysis. Recombinant strains that showed no significant differences ($\alpha = 0.05$) between the two predicted counts were considered stable. These examinations were done in BPB, apple cider, and E-medium immediately after acid adaptation and non-adaptation procedures.

Benzoate and Sorbate Stability Tests

Acid-adapted and nonadapted recombinant cells were tested for plasmid stability in sodium benzoate (Aqua Solutions; Deer Park, TX) and potassium sorbate (Spectrum Quality Products, Inc.; Gardena, CA 90248). This was done by resuspending conditioned cells (pelleted acid-adapted and nonadapted cells) in 1.3mL BPB, apple cider, or E- medium containing 0.1% sodium benzoate and potassium sorbate. The eppendorf tubes were incubated at 30°C for 10 days. The cells were then centrifuged at 10,000 *g* in a microcentrifuge for 3 min. and resuspended in 1.3 mL of BPB. A 0.1 mL aliquot of the BPB was further diluted in 0.9 mL BPB accordingly and spread on TSA-AMP in triplicate. A 1 mL portion of the cell-BPB solution was analyzed by luminometry.

Freeze-thaw Stability Tests

Plasmid stability tests after freezing and thawing involved a preincubation step prior to freezing and thawing cycle. Acid-adapted and nonadapted cells were resuspended in 1.0 mL BPB, apple cider, or E-medium and then incubated in a 37°C water bath for 10 hours. Samples were then removed from the water bath and placed

in a –20°C freezer for 48 hours and thawed in a 4°C water bath for 4 hours. The cells were then centrifuged at 10,000 *g* for 3 min. in the microcentrifuge for 10 min. and resuspended in 1.3 mL of Butterfields phosphate buffer. A 0.1 mL aliquot of the BPB was further diluted in BPB accordingly to allow for spread plating on TSA-AMP in triplicate. A 1 mL aliquot of the original BPB solution was analyzed by luminometry.

Thermal Stability Tests

The conditioned E. coli cells (acid-adapted and nonadapted) were subjected to the highest degree of heat treatment (30 min. at 54°C) that E. coli will be subjected to in this study. After cells were resuspended in 1.3 mL of BPB, apple cider, or E-medium, 0.1 mL aliquots were distributed to 15 sealed Pasteur pipet tips. The pipet tips were placed in a 54°C water bath for 30 min. and then tempered in a 25°C water bath for 1 minute. Samples were analyzed by spread plate method and luminometry. This was done by distributing all samples (1.5 mL total) to an eppendorf tube, washing once in 1.5 mL BPB, and resuspended in 1.5 mL BPB. A 0.1 mL volume was diluted in 0.9 mL BPB accordingly, spread on ampicillin TSA in triplicate, and placed in a 35°C incubator. Luminometry was performed on a 1 mL sample.

Antimicrobial Treatments

Benzoate and Sorbate Treatments

Acid-adapted and nonadapted recombinant cells were treated with sodium benzoate and potassium sorbate to determine survival using plate counts and luminometry. This was done by resuspending cells in 1.3 mL Butterfields phosphate buffer, apple cider, or E-medium containing 0.1% sodium benzoate and potassium sorbate after acid-adapted and non adapted conditioning. The eppendorf tubes were

incubated at 30°C for each day for 10 days. The cells were then centrifuged at 10,000 g in the microcentrifuge for 3 min. and resuspended in 1.3 mL of Butterfields phosphate buffer. A 0.1 mL aliquot of the BPB was further diluted in 0.9 mL BPB accordingly to allow for spread plating on TSA-AMP. A 1 mL portion of the original BPB solution was analyzed by luminometry.

Freeze-thaw Treatments

Plasmid stability tests after freezing and thawing involved a preincubation step prior to freezing and thawing cycle. Acid-adapted and nonadapted cells were resuspended in 1.0 mL BPB, apple cider, or E medium and then incubated in a 37°C water bath for 10 hours. Samples were then removed from the water bath and placed in a –20°C freezer for 48 hours and thawed in a 25°C water bath for 4 hours. The cells were then centrifuged at 10,000 *g* for 3 min. in the microcentrifuge and resuspended in 1.3 mL of Butterfields phosphate buffer. A 0.1 mL aliquot of the cell suspension was further diluted in BPB accordingly to allow for spread plating on TSA-AMP in triplicate. One mL of the original BPB solution was analyzed by luminometry.

Thermal Treatments

The conditioned E. coli cells (acid-adapted and nonadapted) were subjected to increasing time under heated conditions. After cells were resuspended in 1.3 mL of Butterfields phosphate buffer, apple cider, or E-medium, 0.1 mL aliquots were distributed to 15 close ended Pasteur pipet tips. The pipet tips were place in a 54°C water bath for 0, 5, 10, 15, 20, 25, or 30 min. and then tempered in a 25°C water bath for 1 minute. Samples were finally analyzed by spread plate on method TSA-AMP and luminometry. This was done by distributing all samples (1.5 mL total) to an

eppendorf tube, washed once in 1.5 mL BPB, and resuspended in 1.5 mL BPB. A 0.1 mL volume was diluted in 0.9 mL BPB, spread on ampicillin TSA in triplicate, and placed in a 35°C incubator. One mL of the original BPB solution was analyzed by luminometry.

RESULTS AND DISCUSSION

Apple Cider Characteristics

Microbiological and chemical analyses were performed to determine the cider's affect(s) on survival. Microbiological analyses of the cider on orange serum agar revealed an aerobic count of 10^4 CFU/ mL. The cider's aerobic plate count consisted primarily of aerobic organisms, appearing to consist mostly of yeast and mold, existed in the cider. After filtration, the aerobic plate count was 10^2 CFU/ mL with bacteria being the major organisms. The acidity and soluble solids for the cider were pH 3.8±0.2 and 13.2±0.2 °Brix, respectively. These properties (i.e. acidity and soluble solids) of apple cider directly affect survival, as well as influence the effectiveness of treatments. Microorganisms that preexist in the apple cider may competitively exclude the inoculated E. coli O157:H7 and inactivate or inhibit it by generating antimicrobial products. The microbial load of apple cider may affect the effectiveness of the treatments. Chemical properties such as low pH and high soluble solids content, are known to disrupt bacteria by affecting the trans-membrane gradient (Mitchell, 1973). These factors in combination with treatments may have synergistic effects on inoculated bacteria. More specifically, low pH (4.2 to 4.4) is known to enhance the effects of benzoate/sorbate.

Transformant Characteristics

Microscopic qualitative analyses performed before and after transformation confirmed that no obvious differences in morphology existed between wild type and transformed cells. Also, colony morphology and serotypical characterizations were not apparent on media when applying the FDA approved BAM procedures for identifying E. coli O157:H7 on both wild type and transformed cells. Analysis using the API20E strips revealed that no biochemical differences existed between the wild type and transformed organisms as indicated in Appendix A.

The electrotransformation process may modify wild type organisms to produce unintended characteristics. In this study, cell exposure to harsh electric fields may result in cell mutation. However, mutations are more likely to occur after the foreign DNA transfers inside the cell membrane of the target organism. Though the foreign DNA used in this study is an autonomous replicating plasmid, the DNA can potentially recombine with the host genome. This genomic integration would likely create shifts affecting determinants downstream. Since it is impossible to examine the transformed cell for absolute changes, this study took into account a few physical and biochemical alterations that may have resulted from electrotransformation.

Linear Regression Analyses

Linear regression analysis revealed that excellent correlation existed between CFU and RLU as indicated by the high R-square values of 0.97 or higher. The slopes and intercepts for each combination of factors are illustrated in Appendices B and C. Correlation did not seem to be greatly affected by the conditioning of the cells (acid adaptation vs. non-adaptation) or medium used in the study.

Optimal correlation between CFU and RLU is essential to this study as the linear models derived were used to determine counts based on the RLU obtained in later experiments. In providing the linear models, it is important to consider how the matrix (E-medium, buffer, and apple cider) and conditioning of the cells (acid adaptation or non-adaptation) may effect the regression. Therefore, all six combinations of media and conditioning were analyzed through linear regression and used to derive counts for experiments that were associated with a particular combination. For example, linear regression analysis for acid-adapted cells in E-medium were used to correlate RLU to CFU for treatments on acid-adapted cells in E-medium.

Transformation Stability

Observed plate count values were statistically compared (T-tests for unequal variance) with predicted plate count values derived from linear regression models to measure transformation stability. The T-tests conducted for the observed and predicted counts revealed that the predicted values were not significantly different from observed D-values as illustrated in Appendices D, E, and F. This indicated that no loss of expression occurred under the most extreme stress situations, and RLU values were reliable to use in formula's to obtain colony counts.

A concern in using transformed cells in survival studies includes the possibility that the DNA might be shed from the bacterial cell under stressed conditions. Also, expression systems may be adversely affected by stress as the organism may under-express the genetic factors associated with the desired phenotypic response. If *luxABCDE* expression was compromised in this study, linear

regression models would no longer be valid in determining cell counts. Therefore, experiments using the transformed cells were done at the highest stress levels of 30 min., 10 hours, and 10 days of heat, benzoate/sorbate, and freeze-thaw exposure. If correlation of RLU's to CFU showed no significant differences, it was assumed for the purposes of this study that plasmid expression is not affected by the stressed conditions.

Strain Comparisons

The survival on E. coli O157:H7 was observed for strain dependency after treatment. This was done by graphically comparing the strains used in this study for survival over time. Line graphs were developed for heat, benzoate and sorbate, and freeze thaw-treated samples in E-medium, buffer, and apple cider using acid-adapted cells. Values portrayed in the following graphical representations were obtained using RLU measurements that were plugged into the appropriate line equations to derive equivalent cell counts. Slope and intercept values for nonadapted strains are illustrated in Appendices G, H, and I.

Benzoate and Sorbate Treatments

Strain comparisons after benzoate/sorbate treatment on E. coli samples in E-medium, buffer, and apple cider revealed strain specific survival trends. According to RLU-derived counts using acid-adapted bacteria (Figure 1), strain A and C samples indicated similar survival trends, with cell numbers showing approximately a 1-log reduction in 5 days between day 3 and 8 and an overall decline of 1.5-logs. Strain B appeared to be most resistant as only a 1-log reduction was exhibited throughout the 10 day period. The non-O157 control was most sensitive in E-medium with close to a

2-log reduction in 5 days between day 3 and 8. Non-O157 counts showed a final 3-log reduction over the 10 day period.

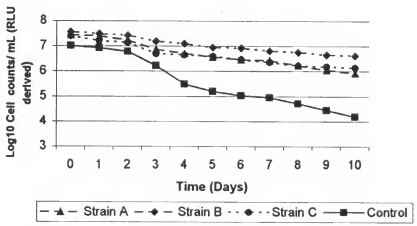

Figure 1. Relative light unit-derived counts for benzoate/sorbate-treated acid-adapted organisms in E-medium.

Benzoate/sorbate-treated buffer samples showed similar viability trends as samples in E-medium (Figure 2). Strain B survived the best with a 1-log decline in 5 days between days 3 and 8 and an overall decline of close to 1.5-logs. Strains A and C exhibited comparable sensitivities with a 1-log reduction in 3 days between days 3 and 8 with final decline nearing 2.5-logs over the 10 day period. Benzoate/sorbate-treated non-O157 E. coli showed least viability over time with close to a 2-log reduction in 3 days between days 2 and 5 and close to a 3.5-log decrease in activity over the 10 day period.

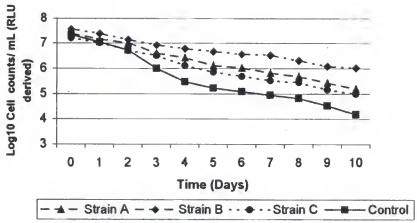

Figure 2. Relative light unit-derived counts for benzoate/sorbate-treated acid-adapted organisms in buffer.

Benzoate/sorbate seemed to be most effective in apple cider as indicated in Figure 3. Strain A showed the least sensitivity with a 1-log reduction in 5 days between day 3 and 8 and a 10 day decrease of nearly 2-logs. Strain B and C samples displayed similar survival indicated by a 1-log reduction in 4 days between day 2 and 6 and a final decrease approaching 2.5-logs. The non-O157 control exhibited the least resistance with a 2-log reduction in 3 days between day 1 and 4 and an overall decline close to 3-logs over the 10 day period.

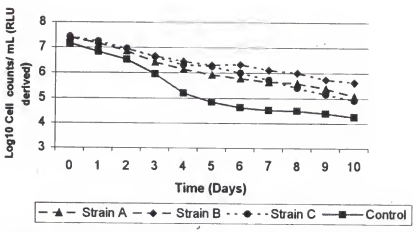

Figure 3. Relative light unit-derived counts for benzoate/sorbate-treated acid-adapted organisms in apple cider.

Benzoate/sorbate treatment showed between a 1.5 to 3-log reduction depending on the strain and matrix type. It was found that E. coli O157:H7 showed maximum declines of approximately 2.5-logs in apple cider. This disagrees with the findings by Fisher and Golden (1998) and Zhao et al. (1993) et al. that showed decrease of over 5-logs before 10 days. This is surprising especially since the temperature in this study was five degrees higher (30°C) than their study (25°C). One major difference between the studies is that this study involved the use of acid adapted cells. Other differences may be due to varying cider characteristics (pH, brix, etc), strain type, and storage conditions. Benzoate/sorbate treatment appears to be most effective between days 3 and 8. The activity of the preservatives is limited during days 1 and 2, as the organism die-off at these times indicating a slight lag effect. This lag effect is consistent with findings by Fisher and Golden (1998) showing a lag effect for the first two days of treatment. This may occur as the preservative activity is time dependent and simply did not have enough time to take

full effect. After day 8, benzoate/sorbate activity is again compromised perhaps because the preservatives have lost optimal activities or the organisms have adapted to the preservatives by generating protective proteins. Another explanation for this tailing is that resistance variability within the strain may exist

Strain B was consistently the most resistant to benzoate/sorbate treatment while strains A and C were effected relatively equally by the treatment. The control E. coli was generally the most sensitive to the treatment in all matrices. Strain resistance may be a result of defense responses by the organisms that protected the organism from the benzoate and sorbate activity. Since benzoate and sorbate are known to target the cytoplasmic membrane and cellular enzymes, the more resistant cells may possess membrane stabilizers, such as proteins, that disallow membrane disruption or penetration (Kruk and Lee, 1982).

The matrix type seemed to effect organism viability with organisms surviving the best in E-medium followed by apple cider and buffer. The constituents or general nature of apple cider and buffer may increase the activity of the preservatives, while those in E-medium are more conducive for survival. The activity of sodium benzoate and potassium sorbate is known to be greatest in low pH environments. Levels of undissociated benzoate and sorbate increase with increasing acidity. The presence of undissociated benzoate and sorbate allows for cell membrane penetration with eventual damage to the cell. For this reason, high pH E-medium and buffer were not expected to have as great of an effect. In addition to the influences that the matrix has on the benzoate/sorbate, the constituents in the matrices may enhance organism

resistance by allowing the bacteria to utilize them for their defense mechanisms against cellular damage.

Freeze-thaw Treatments

Data for freeze-thaw-treated acid-adapted strains A, B, C and E. coli control in all three matrices was plotted and analyzed to assess possible strain variation. In E-medium, strain C showed the most resistance after the 10 hour treatment with close to a 3-log reduction over the 10 hour period (Figure 4). Strains B, C, and the non-O157 control exhibited similar sensitivities with cell viability reducing close to 5.5-logs after 10 days. Of these three organisms, strain A appeared to have greatest resistance to freeze-thawing, followed by the control and strain B.

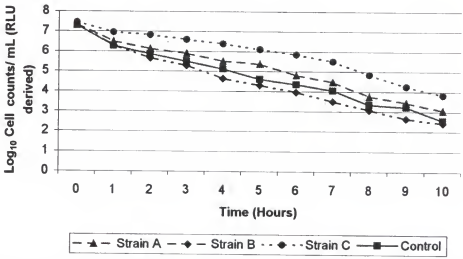

Figure 4. Relative light unit-derived counts for freeze-thaw-treated acid-adapted organisms in E-medium.

When frozen and thawed in buffer, strain C again appeared to be more resistant with a final reduction of nearly 4-logs (figure 5). Survival trends for Strain A and the control strain were nearly identical with declines close to 4.5-logs. Strain

B appeared to offer the least resistance to the treatment with over a 5.5-log decrease in activity over the time period.

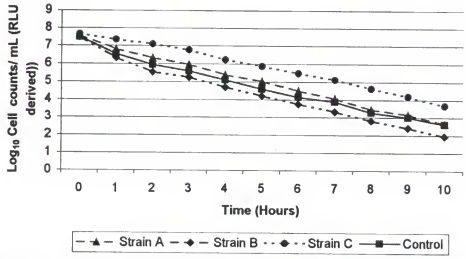

Figure 5. Relative light unit-derived counts for freeze-thaw-treated acid-adapted organisms in buffer.

More disparity between the organisms existed after freeze thaw treatment in apple cider (figure 6). Consistent with the other media, strain C exhibited the greatest survival in cider with a 4-log decline over 10 days. The control sample, showed slightly less resistance than Strain C though counts also ended with a 4-log reduction after the 10 hours as cell numbers dropped lower than those for Strain C after day 2. Treatments on strain A resulted in close to a 5-log decrease in cell numbers while strain B exhibited greater sensitivity with over a 5-log drop in viability.

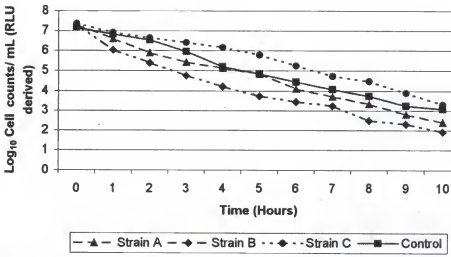

Figure 6. Relative light unit-derived counts for freeze-thaw-treated acid-adapted organisms in apple cider.

Freeze thawing seemed to cause a steady decrease in cell numbers without sharp drops in counts throughout the time of treatment. This steady decline is consistent with a study by Ingham and Uljas (1998) on freeze-thawing effects on Salmonella typhimurium in apple cider. The 6 hour 4.5-log reduction of E. coli O157:H7 in apple cider also agrees with Ingham and Uljas findings for S. typhimurium.

Strain B exhibited the most resistance surviving the best in all media. Strain A and the control showed similar patterns of decline with strain showing greater resistance of the two. Strain C showed the least resistance to freeze thaw in all media. Strain resistance to the treatment may be due to the ability to endure both the freezing and thawing stages. The process effects may affect the organism in several ways depending on freezing and thawing rates. The initial cold shocking diminishes cell membrane permeability and metabolically injures the cell. This effect is most likely the major source of inactivation in this study, as cells that were held at optimal

growth temperatures (35°C) throughout the 10 hour period, were suddenly subjected to freezing temperatures. This quick change from mesophilic temperatures to freezing temperature most likely heightens the deleterious effects of cold shock. Also, during freezing microorganisms are subjected to osmotic shock and mechanical damage by crystal formation. Intracellular ice crystallization results in pH and ionic strength changes that can cause inactivation of enzymes and the denaturation of proteins, DNA and RNA. A stabile cytoplasmic membrane possibly consisting of osmotically stabile proteins may reduce the freeze damage to the cell by remaining functional during the process. Intracellular resistance to freezing and thawing involves generating amino acids that allow the cytoplasm to maintain water activity for metabolic processes. Proline has been studied to function this way for E. coli according to Grothe et al. (1986).

The organisms exhibited greatest survival in E-medium, followed by apple cider and buffer. The suspension medium is known to play a major role in the survival of microorganisms during freezing and thawing as it influences the length of time that the bacterial cell undergoes osmotic stress. A high osmotic solution, such as apple cider, allows for a high freezing point and hence a long exposure to osmotic damage. This is consistent with the relatively low survival in apple cider found in this study. E-medium would be expected to allow for higher inactivation as it also would result in high osmotic damage. Conversely, the E-medium resulted in relatively high survival possibly because the matrix allowed for good cell recovery after the freeze damage. The organisms in buffer solution was expected show the greatest survival because of the relatively low osmotic pressure associated with the

media. However, the relatively low viability in buffer may be due to crystal formation associated with the constituents in the buffer solution (Jay, 1998b).

Thermal Treatments

Strain variation was assessed for cells in E-medium, buffer, and apple cider after thermal treatment. In E-medium, strain A and B showed similar trends as both exhibited highest resistance with a 2.5-log decrease in 15 min. between time 5 and 20 min. and close to 4-log drop over the 30 minute period (Figure 7). It appeared that strain C showed slightly greater resistance than strain A as values for the two were relatively equal with strain C indicating slightly higher overall viability especially at 15 and 25 minute time points. Of the O157 cells, strain B showed the least resistance as cell numbers dropped 4.5-logs in 15 min. between the 5 minute and 20 minute time point. Strain B showed close to 6-log decrease over the 30 min. The control showed most sensitivity to thermal treatment overall, as survival declined nearly 5-logs for 15 min. between 5 and 20 min. and decreased 7-logs over the 30 minute trial period.

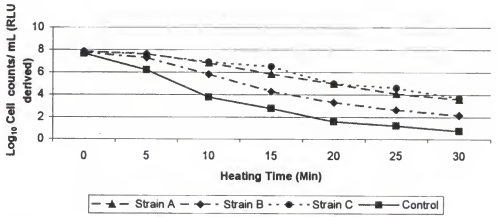

Figure 7. Relative light unit-derived counts for heat-treated acid-adapted organisms in E-medium.

Heat-treated strains in buffer showed greatest decline in cell counts between time 10 and 20 min. (Figure 8). Strain B exhibited greatest survival with counts after 10 min. between 10 and 20 declining 2-logs. Strain A showed slightly less resistance than strain B with cell numbers declining slightly less than 2-logs in 10 min. between 10 and 20 min. Both strains showed a 5-log drop over the 30 minute period. Strain B was more sensitive to heat treatment than strain A and C as counts declined close to 4-logs in 10 min. between 10 and 20 min. with over 6-log reduction in 30 min. The nonO157 control was clearly the most sensitive but displayed a unique pattern as cell numbers dropped sharply as early as 5 min. with cell counts stabilizing, showing slight reduction after 10 min. The control dropped over 6-logs over the 30 minute period.

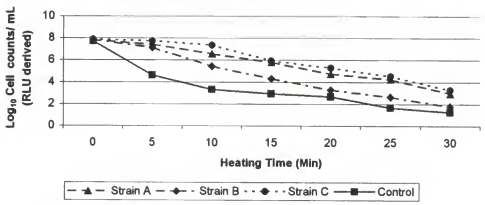

Figure 8. Relative light unit-derived counts for heat-treated acid-adapted organisms in buffer.

Samples that were heated in apple cider showed a sharp but steady decline over the 30 minute time period (Figure 9). As with samples in E-medium strain A and C showed strikingly similar patterns of survival with strain A exhibiting slightly greater resistance. These strains declined approximately 4-logs in 15 min. between the 5 and 25 min. time point with final reductions of close to 6-logs. Strain B exhibited less survival with a decline of slightly over 4-logs in 15 min. between the 5 and 20 minute time points. Strain B showed close to a 7--log reduction over the 30 minute time period. Consistent with the other heat-treated samples the control dramatically declined 5-logs in 15 min. between the 5 and 20 minute time period with no survival at 30 min.

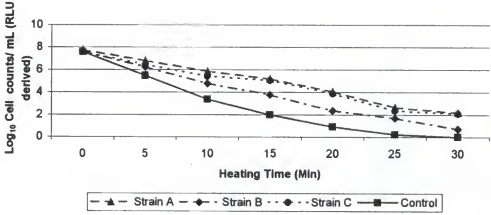

Figure 9. Relative light unit-derived counts for heat-treated acid-adapted organisms in apple cider.

Thermal treatment resulted in decreases from 3.5 to 7-logs depending on the strain and matrix type. The 6-log drop in apple cider is consistent with findings from Splittstoesser et al. (1995) that showed approximately 6-log decline in apple juice at 55°C. However, Splittstoesseret al. did not use acid-adapted cells in their study. Acid-adapted E. coli O157:H7 showed a 4.5-log drop after 30 min. at 56 C according to a study by Rowe and Kirk (1999). Ryu and Beuchat (1999) found that cell count reduction dependent on strain type as acid-adapted E. coli O157:H7 showed between 1 to 4.5-log decrease after 30 min. at 54 C depending on the strain.

The organisms subjected to heat experienced a 10 minute lag period and a leveling of cell counts after 25 min. This same effect is exhibited in data from studies by Buchanon and Edelson (1996) and Rowe and Kirk (1999). The early lag period may be due to insufficient heating time for inactivation effects to occur. Adaptation to heat may take place after the 25 min. period thus resulting in the leveling of cell numbers. Also, variability in heat sensitivities within the solution of the same strain

type may cause this slight tailing. Other contributing factors include clumping of cells and adhesion to the heating vessel.

Results were variable when comparing strains between the different matrices. Strains A or C appeared to be the most resistant to heat depending on the matrix. Strain B showed more sensitivity than strains A and C in all media while the control E. coli was the most sensitive to the treatment. Bacteria are affected by heat in many different ways including the denaturation of nucleic acids, structural proteins, and enzymes (Gould, 1989). However, mild heat treatments such as that applied in this study, have been studied to affect cytoplasmic membrane. Therefore resistance to heat may deal with the stability of the cytoplasmic membrane. Recent studies have indicated that the heat shock proteins generated by E. coli serve to stabilize the cell membrane throughout the heating event (Hightower, 1991). Therefore, the resistance to heat by strain A and C may be due to a more stabile membrane.

When comparing matrices, E-medium was most conducive for bacterial resistance followed by buffer, and apple cider. The type of suspension medium has a profound effect on heat-treated samples as extreme pH contributes to heat inactivation of bacteria, while the presence of organic material and proteins protects cells from thermal injury (Condon and Sala, 1992; Jay, 1998a). Carbohydrates and sometimes salts, depending on the kind and concentration, increase heat resistance as they reduce the water activity inside the organism (Corry, 1974). Though the sugar level (°Brix) in apple cider is high, the acidic nature of the matrix was most likely a major contributor to the low survival observed in this study. This may due to slight denaturation of proteins or enzymes from the heat, exposing them to the destructive

hydrogen protons associated with the acid. Samples heat-treated in E-medium showed the greatest survival perhaps as the nutrients associated with the media are helpful in cell resuscitation.

Sublethal Injury

Sublethally injury was assessed by comparing actual colony-forming unit counts, obtained from the plate count technique, with predicted cell counts, using RLU values obtained through luminometry. To derive predicted counts, RLU values were integrated into linear equations from regression analyses that correlated RLU's with CFU's. Sublethally injured cells were assumed to exist if the RLU-derived counts (dCFU) are greater than plate counts (CFU). General trends were observed using line graphs that compared the two counts. Also, D-values were formulated for RLU-derived count and plate count data using linear regression equations that yielded a correlation of $R = 0.90$ or greater. RLU-derived D-values and plate count D-values were statistically compared for each strain using T-test assuming unequal variances Appendices J, K, and L. Finally, RLU-derived count and Plate count averages for E. coli O157:H7 were calculated and compared for each matrix.

Benzoate and Sorbate Treatments

A comparison between plate counts and RLU-derived counts in apple cider and buffer is provided in Figure 10. The RLU-derived counts are generally higher than plate counts for the apple cider and buffer samples. The apple cider RLU-derived count line appeared to stay the same length above the apple cider plate count line throughout the treatment period. However, the buffer plate counts and RLU-

derived counts are nearly the same until day 6 when separation between the two is more apparent.

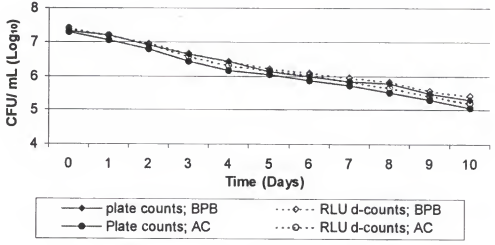

Figure 10. Plate counts versus RLU-derived counts using acid-adapted cells after benzoate/sorbate treatment.

A comparison of D-values obtained for each sample series showed that D-values obtained from RLU-derived counts were nearly always greater than those obtained from plate counts in apple cider. A comparison of plate counts and RLU-derived counts for acid-adapted strains in apple cider is given in Figure 11. Though no significant differences between D-values were found for these strains, the RLU-derived counts were greater than plate counts according to the raw data. The control E. coli exhibited the greatest positive difference when subtracting CFU from dCFU with a value of 0.07 days. This difference was closely followed by strain A, B, and C that showed positive differences of 0.04, 0.02, and 0.01 days., respectively.

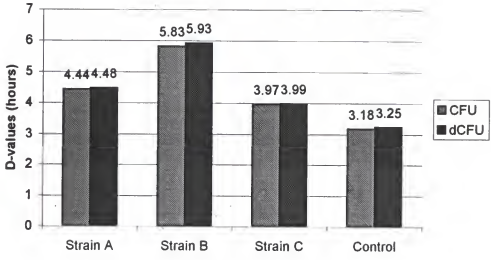

Figure 11. A comparison of plate count (CFU) and RLU-derived count (dCFU) D-values for apple cider samples after benzoate/sorbate treatment on acid-adapted strains.

A matrix comparison of D-values also showed that RLU-derived counts were greater than plate counts. An illustration of matrix D-value comparisons based on average RLU-derived counts and plate counts for acid-adapted E. coli O157:H7 is given in Figure 12. After subtracting average E. coli O157:H7 CFU from dCFU it was determined that E-medium and buffer showed similar D-value differences of 0.28 and 0.21 days, respectively. Apple cider showed much less difference with a value of 0.04 days.

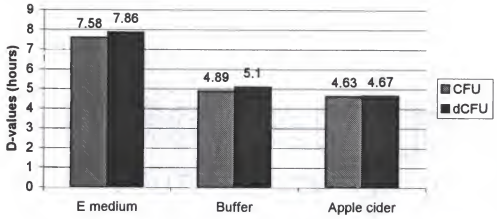

Figure 12. Average acid-adapted E. coli O157:H7 colony-forming unit counts (CFU) versus relative light unit counts (dCFU) D-values for all matrices after benzoate/sorbate treatment.

Freeze–thaw Treatments

The RLU-derived counts were greater than plate counts for nearly all samples that were freeze thawed. Average E. coli O157 RLU-derived counts were greater than average E. coli O157 plate counts for buffer and apple cider samples (Figure 13). It appeared the difference increased with time of treatment, as the difference at 10 hours was the greatest for both apple cider and buffer samples.

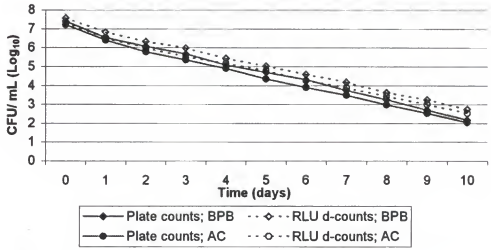

Figure 13. Plate counts versus RLU-derived counts using acid-adapted cells after freeze-thaw treatment.

D-values using RLU-derived counts were generally greater than those developed using plate counts. When comparing adapted strain dCFU to CFU for apple cider samples, differences were nearly the same (Figure 14). The control showed the largest difference of 0.22 hours, followed by strain A, C, and B with values of 0.2, 0.19, and 0.16, respectively. Statistical analyses indicated that a significant difference ($\alpha = 0.05$) between CFU and dCFU existed for strains B, C, and the control.

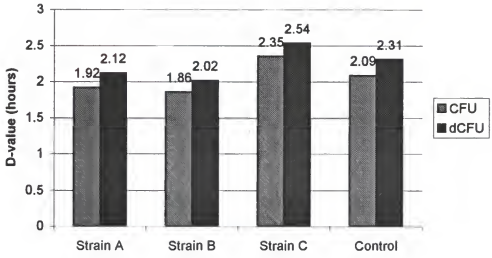

Figure 14. A comparison of plate count (CFU) and RLU-derived count (dCFU) D-values for apple cider samples after freeze-thaw treatment on acid-adapted strains.

RLU-derived count D-values were greater than plate count D-values for all matrices after freeze thaw treatment (figure 15). After calculating the difference between dCFU and CFU for each matrix, apple cider and E-medium showed greater differences with values of 0.27 and 0.29 min., respectively. Buffer showed the least difference with a difference value of 0.08 min.

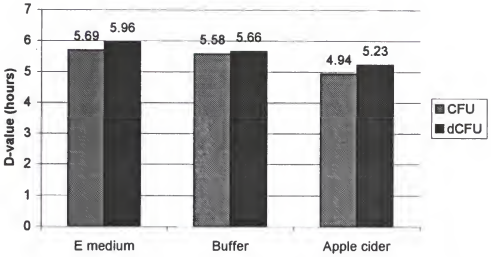

Figure 15. Average acid-adapted E. coli O157:H7 colony-forming unit counts (CFU) versus relative light unit counts (dCFU) D-values for all matrices after freeze-thaw treatment.

Thermal Treatments

The RLU-derived counts were greater than plate counts for nearly all samples after thermal treatment. The difference between plate counts and RLU-derived counts was greatest for buffer samples over time of treatment than apple cider samples (Figure 16). The difference between plate counts and RLU-derived counts in buffer samples appeared to be consistent throughout the time period unlike with apple cider samples that showed the largest difference between time 10 and 25 min.

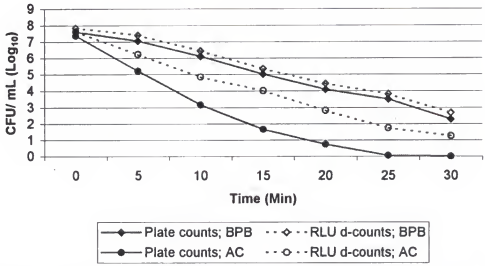

Figure 16. Plate counts versus RLU-derived counts using acid-adapted cells after thermal treatment.

The D-values that were obtained using the data from RLU-derived counts and plate counts showed differences in favor of the RLU-derived counts after thermal treatments. Strain A showed the largest difference between dCFU and CFU D-values with a D-value difference of 0.77 min., while strains B and C showed the same dCFU-CFU with D-value differences of 0.11. The control E. coli dCFU-CFU difference was slightly less at 0.09. A significant difference (α =0.05) was only observed for strain A.

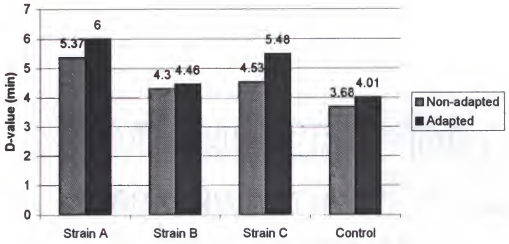

Figure 17. A comparison of plate count and RLU-derived count D-values for apple cider samples after thermal treatment on acid-adapted strains.

A comparison of matrix associated D-values, using RLU-derived count and plate count averages of E. coli O157:H7 strains, showed that the dCFU were greater than the CFU for all matrices (Figure 18). Results from using acid-adapted strain averages indicated that the largest difference at 0.29 min. was obtained for apple cider samples, followed closely by E-medium showing a slightly lower dCFU–CFU difference of 0.27 min. The buffer samples showed the least dCFU-CFU difference with a D-value of 0.08 min.

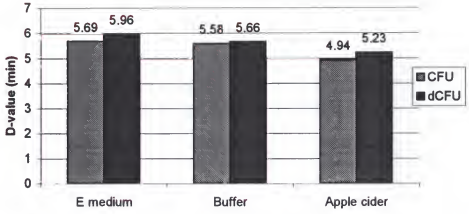

Figure 18. Average acid-adapted E. coli O157:H7 colony-forming unit counts (CFU) versus relative light unit counts (dCFU) D-values for all matrices after thermal treatment.

Sublethally injured cells existed in most treated samples as RLU-derived counts are nearly always greater than plate counts for all treatments. The linear patterns for buffer and apple cider illustrated in figures 11, 13, and 16 may represent repair mechanism activity. The consistent differences may indicate that repair mechanisms are simply unable to repair cell damage. Matrix line patterns that exhibited increasing differences over time could represent progressive loss of cellular repair as treatment time increases. An ineffective cell repair system may be completely inactivated from damage or simply unable to deal with the injury load.

D-values were valuable in measuring overall trends of survival over treatment time, especially since R^2 for all samples were above 0.90. D-value comparisons of plate counts vs. RLU-derived counts exhibited variable calculated differences for all strains. Significant differences were mostly achieved by freeze-thaw-treated strains, as the cells may have been static after the treatment. Overall, strain A D-values showed the larger differences between plate counts and RLU-derived counts after all

treatments. The control showed large D-value differences after freeze-thaw but lowest after treatment with heat. Strains B and C gave marginal differences after all treatments. These calculated differences may be an indication of how each strain deals with antimicrobial stress. Strains that show a greater positive difference when subtracting plate counts from RLU-derived count may carry more advanced repair mechanisms, as they are able to maintain viability for future full recovery from the damage. The effectiveness of repair mechanisms may be dependent on the type and degree of treatment as well as suspension media type that allows for potential recovery.

Matrix comparisons of plate count and RLU-derived D-values may give an indication on the matrix effect on sublethally injured cells. According to the data obtained in this study, samples in apple cider and E-medium gave the largest differences except for benzoate/sorbate-treated samples when apple cider showed the least difference. A large difference between plate counts and RLU-derived counts for a matrix is suspected to be a good indication that the matrix is favorable for maintaining sublethally injured cells. The buffer samples mostly showed the least differences between plate counts and RLU-derived counts and therefore may not be favorable for sublethally injured cells. These results are expected as the apple cider was predicted to be conducive for the cells to be sublethally injured due to the osmotic and acidic nature of the matrix. The slight indication of sublethal injury in E-medium was also expected, as it is likely that the nutrients associated with E-medium allowed for sublethally injured cells to repair themselves more readily. These results

suggested that nutrients associated with the E-medium might have contributed to full recovery of the cell, therefore minimizing differences.

Cross Protection

Cross protection was assessed by comparing acid-adapted E. coli strains with nonadapted strains. If acid-adapted counts were significantly greater ($\alpha = 0.05$) than nonadapted counts it was assumed that cross protection existed. A statistical analysis (T-test unequal variances) revealed that significant differences did exist between acid-adapted and nonadapted cell D-values for most strains (Appendices M, N, and O). Raw D-value data for each conditioned strain was compared to illustrate general trends.

Benzoate and Sorbate Treatments

Acid-adapted counts were nearly always greater than nonadapted counts after benzoate/sorbate treatment. A comparison of acid-adapted and nonadapted cells using E. coli O157 strain count averages in buffer and apple cider is provided in figure 19. This figure illustrates that the difference between adapted and nonadapted cells in apple cider was generally greater than the difference between adapted and nonadapted cells in buffer. Also, the difference between acid-adapted and nonadapted cells in both media increased with increasing treatment time, especially for apple cider samples.

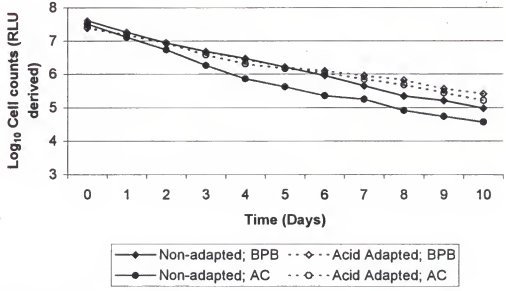

Figure 19. A comparison of average RLU-derived counts of nonadapted and acid-adapted cells.

When comparing D-values derived for acid-adapted cells with nonadapted cells it was determined that the acid-adapted D-values were generally greater. An illustration of acid-adapted vs. nonadapted D-values in E-medium after benzoate/sorbate treatment is given in figure 20. This figure shows that D-value difference between acid-adapted and nonadapted strain C was the greatest with a D-value difference of 4.14 days. The adapted – nonadapted difference for strain A was next highest with a D-value of 2.25 days, followed by Strain B and the control showing D-value differences of 1.97 and 0.45 days, respectively. A two-tailed statistical analysis revealed that acid-adapted cells showed significantly higher (α = 0.05) D-values than nonadapted cell D-values for all strains in E-medium.

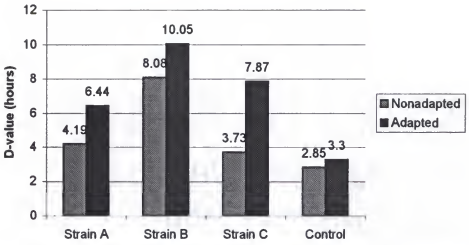

Figure 20. A comparison of acid-adapted and nonadapted D-values (RLU-derived count) in E-medium after benzoate/sorbate treatment.

In buffer solution, all strains showed similar differences when subtracting nonadapted from acid-adapted cell D-values (figure 21). Strain A showed the greatest adapted – nonadapted difference of 1.41 days, followed closely by strain C and the control with differences of 1.31 and 1.16 days, respectively. Strain B showed the least difference with an adapted- nonadapted D-value difference of 1.03 days. A two-tailed statistical analysis revealed that acid-adapted cells showed significantly higher ($\alpha = 0.05$) D-values than nonadapted cell D-values for all strains in buffer.

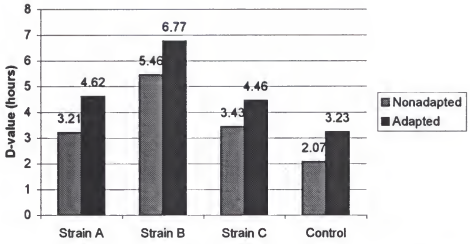

Figure 21. A comparison of acid-adapted and nonadapted D-values (RLU-derived count) in buffer after benzoate/sorbate treatment.

D-value comparisons between adapted and nonadapted RLU-derived counts for samples in apple cider showed that the adapted cells showed greater D-values than nonadapted cells (figure 22). The greatest difference was observed for Strain B with a D-value of 1.6 days. Strain A, C and the control E. coli showed similar adapted-nonadapted differences of 1.28, 1.03, and 1.1 days, respectively. A two-tailed statistical analysis revealed that acid-adapted cells showed significantly higher (α = 0.05) D-values than nonadapted cell D-values for all strains in apple cider.

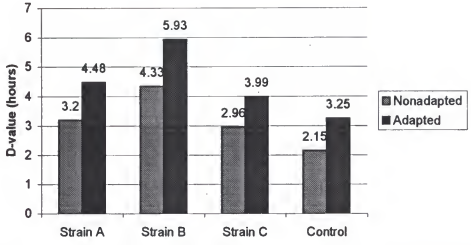

Figure 22. A comparison of acid-adapted and nonadapted D-values (RLU-derived count) in apple cider after benzoate/sorbate treatment.

Using the RLU-derived counts of E. coli O157:H7 strains averaged together, nonadapted and adapted D-values were established for E-medium, buffer, and apple cider (figure 23). The adapted–nonadapted difference was greatest for E-medium samples while buffer and apple cider samples gave similar D-value differences of 1.29 and 1.27 days, respectively

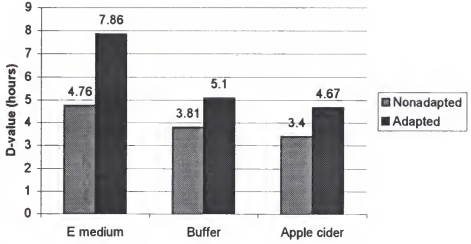

Figure 23. A comparison of acid-adapted and nonadapted D-values (RLU-derived count) for each matrix after benzoate/sorbate treatment.

The results obtained from benzoate/sorbate-treated samples were fairly consistent with a few exceptions. The linear plots comparing acid-adapted and nonadapted E. coli O157:H7 illustrated in figure 19., are irregular for both buffer and apple cider matrices. The increasing difference for both samples may be due to an adaptive response by the organisms that increased with time.

Strains A and B appeared to show the most cross protection against benzoate and sorbate for all matrices. Overall, Strain C showed moderate cross protection with exception of samples in E-medium. The control generally exhibited the least cross protection when exposed to benzoate/sorbate treatment. Goodson and Rowbury (1989) found that acid-adapted cells survived well in benzoic or sorbate. The difference in cross protection between strains may be due to strain specific stress responses. Garren et al., (1998) observed that certain adapted strains of E. coli O157:H7 showed greater cross protection against sodium lactate than other strains. Adaptive proteins generated by the organism serve as a physical or ionic barriers to benzoate and sorbate. Also, structural proteins that have hydrophobic and negatively charged side chains from dissociated benzoate and sorbate. A negatively charged protein may repel benzoate and sorbate that exist in the dissociated form. Another possible protective mechanisms, includes adaptive production of proteins that buffer the intracellular increase in proton concentration that results as the weak acids pass through the membrane.

E-medium appeared to be the most conducive for cross protection after benzoate/sorbate treatment. Perhaps the nutrients associated with the matrix were utilized by the organism for protective responses. This contradicts findings that cross

protection increase under starvation conditions. The apple cider and buffer may have been insufficient for this purpose.

Freeze-thaw Treatments

An analysis of acid-adapted vs. nonadapted cells after freeze-thaw treatment showed variable results. A comparison of acid-adapted and nonadapted cells in buffer and apple cider indicated a very little difference existed between adapted and nonadapted cells in buffer with increasing time of treatment (Figure 24). In contrast, a fairly consistent adapted–nonadapted difference was observed for apple cider with greater differences towards the end of treatment.

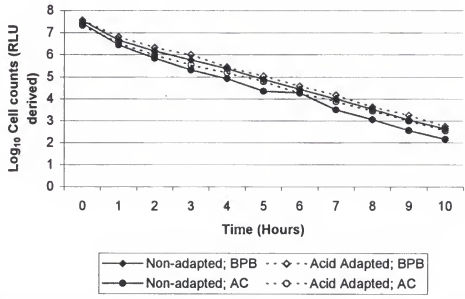

Figure 24. A comparison of average RLU-derived counts of nonadapted and acid-adapted cells.

D-value comparisons between adapted and nonadapted were made using RLU-derived counts after freeze-thaw treatment. A comparison made in E-medium shows variable results as nonadapted strain A cells are greater than adapted cells by a

difference of 0.08 hours, while no differences were observed for the control sample (Figure 25). However, strain B and C showed similar acid-adapted–nonadapted differences of 0.18 and 0.22 hours, respectively. Only acid-adapted were only significantly higher ($\alpha = 0.05$) than nonadapted cells for strain C.

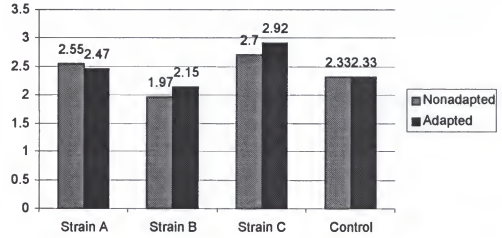

Figure 25. A comparison of acid-adapted and nonadapted D-values (RLU-derived count) in E-medium after freeze-thaw treatment.

Adapted–nonadapted differences in buffer also showed positive and negative differences (Figure 26). Strain A and the E. coli control exhibited a difference of -0.09 and -0.08 hours when subtracting adapted from nonadapted D-values. Strain B and C only showed an adapted–nonadapted D-value difference of 0.03 and 0.12 hours, respectively. None of these strains showed significant differences ($\alpha = 0.05$) between acid-adapted and nonadapted D-values.

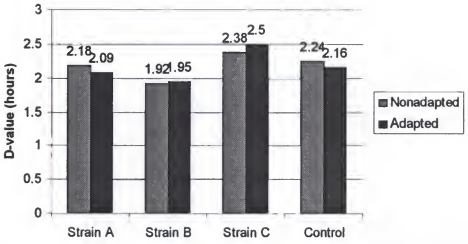

Figure 26. A comparison of acid-adapted and nonadapted D-values (RLU-derived count) in buffer after freeze-thaw treatment.

Freeze-thawed samples in apple cider showed greater adapted D-values than nonadapted samples (Figure 27). Strain C showed the greatest adapted–nonadapted difference of 0.36 hours followed closely by the E. coli control showing 0.31 hour difference. A D-value difference of 0.2 hours was observed for Strain B when subtracting nonadapted cells from acid-adapted cells. Strain A showed the least difference between adapted and nonadapted cells with a value of 0.02. A significant difference (two tailed; $\alpha = 0.05$) between adapted and nonadapted cells was observed for strain B, C, and the control strain.

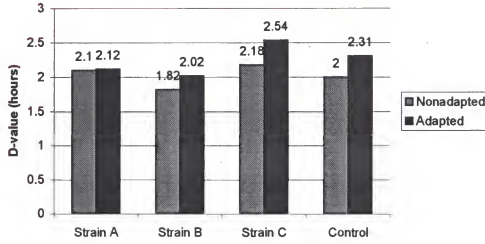

Figure 27. A comparison of acid-adapted and nonadapted D-values (RLU-derived count) in apple cider after freeze-thaw treatment.

Little differences existed when comparing acid-adapted and nonadapted D-values for different matrices (Figure 28). No D-value difference was observed for E-medium samples while buffer samples only showed a 0.02 hour difference. Apple cider adapted–nonadapted difference was greatest in apple cider with a calculated difference of 0.19 hours.

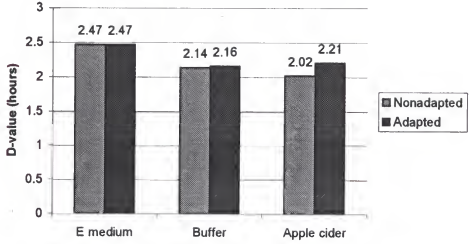

Figure 28. A comparison of acid-adapted and nonadapted D-values (RLU-derived count) for each matrix after freeze-thaw treatment.

Comparisons between acid-adapted and nonadapted cells after freeze thaw treatment were highly inconsistent. As with benzoate/sorbate-treated samples line plots were mixed as the apple cider data showed possible cross protection after a certain period of time, while buffer was more consistently protective throughout the time period.

Cross protection did not appear to exist for certain strains after freeze-thaw treatment as few significant differences between acid-adapted and nonadapted cells existed. Strains B and C showed the most cross protection for all matrices, while strain A and the control strain showed the least and in some cases no cross protection after freeze-thaw treatment. Again this may be an indication of strain specific cross protection. Protection may be in the form structural stability in the cell membrane, possibly as a result of membrane modifications. The stabile membrane is able to maintain integrity and resist shearing in the presence of crystals that may otherwise do mechanical damage. Also, cross protection may be from factors that allow the cell to endure extreme pH and osmotic conditions. Jenkins et al. (1990) found that osmotically adapted E. coli showed greater survival against osmotic challenge (0.35M NaCl) than nonadapted organisms. Conversely, Ryu and Beuchat (1999) observed that no significant differences existed (α = 0.05) between acid-adapted and nonadapted E. coli O157:H7 that were subjected to high osmotic stress (high salt conc.) and dehydration conditions (a_w = 0.70) in beef powder. Proteins generated as a protective response may serve as buffer agents in the cytoplasm or are as active pumps for the regulation of membrane gradient regulation. It was discovered that 22 proteins are generated from osmotic stressed E. coli including 5 starvation proteins

and 2 heat shock oriented proteins (Jenkins, 1990). Sato et al., (2000) found that the outer membrane proteins ompC and ompF were generated by E. coli at increased osmotic levels. Leyer and Johnson (1993) found that only ompC was present in osmotically stressed cells, however the hydrophobicity of the cell surface increased.

The apple cider and buffer seemed to be more conducive for cross protection. This contrasts findings after benzoate/sorbate treatment. However, reasons for the optimal protection in these matrices may be due to the initiation of starvation stress proteins.

Thermal Treatments

Acid-adapted cell counts were generally greater than nonadapted cell counts after thermal treatments. A comparison of acid-adapted and nonadapted cells in buffer and apple cider showed that the RLU-derived counts are mostly greater throughout the treatment time period (Figure 29). In buffer, the adapted cells are consistently higher than nonadapted cells. Adapted cell numbers in apple cider were actually lower than nonadapted counts until 15 min. was reached. Adapted numbers after 15 min. were greater than nonadapted.

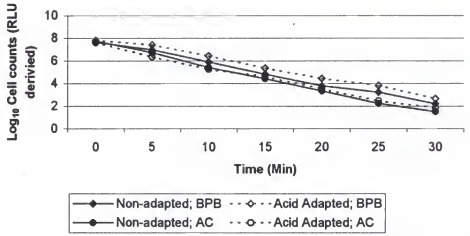

Figure 29. A comparison of nonadapted and acid-adapted cells (RLU-derived count) after thermal treatment

D-values for adapted and nonadapted cells were compared in E-medium (Figure 30). The difference in D-values after subtracting nonadapted from adapted cells was greatest for strain C with a D-value difference of 0.72 min. Strain A showed an acid-adapted –nonadapted difference of 0.22 min. while the control showed a difference of 0.06 min. Strain B acid-adapted D-values were lower than nonadapted cells showing a difference D-value of 0.02 min. A two tailed statistical analysis showed that acid-adapted cells were significantly higher ($\alpha = 0.05$) for all strains with exception to strain B.

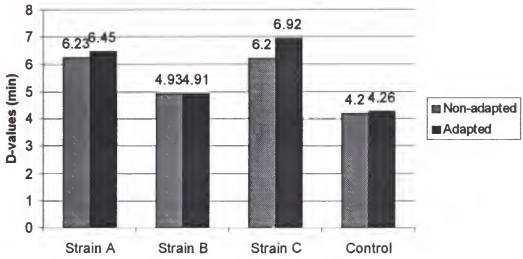

Figure 30. A comparison of acid-adapted and nonadapted D-values (RLU-derived count) in E-medium after thermal treatment.

In buffer, the control strain showed the greatest acid-adapted – nonadapted difference with a difference D-value of 1.19 min. followed by strain A with a D-value difference of 0.34 min. (Figure 31). The D-value differences for strains B and C were similar with D-values of 0.22 and 0.24 min., respectively. A two tailed statistical analysis showed that acid-adapted cells were significantly higher ($\alpha = 0.05$) for all strains with exception to strain C.

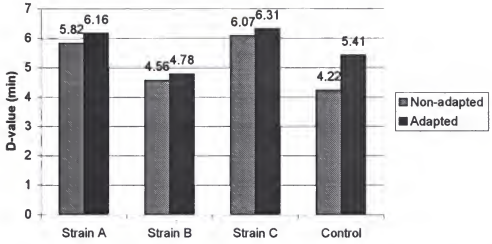

Figure 31. A comparison of acid-adapted and nonadapted D-values (RLU-derived count) in buffer after thermal treatment.

D-values established for acid-adapted cells in apple cider were subtracted from nonadapted cells (Figure 32). The difference was greatest for strain C with an acid-adapted-nonadapted difference of 0.95 min. Strain A showed the next highest difference with a difference D-value of 0.63 min. while the control and Strain B exhibited differences of 0.33 and 0.16 min., respectively. A two tailed statistical analysis showed that acid-adapted cells were significantly higher ($\alpha = 0.05$) for all strains with exception to strain B.

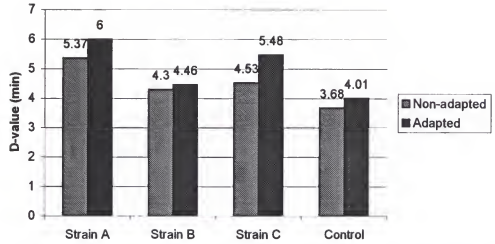

Figure 32. A comparison of acid-adapted and nonadapted D-values (RLU-derived count) in apple cider after thermal treatment.

A comparison of acid-adapted–nonadapted D-value differences in different matrices showed that apple cider and E-medium showed similar differences of 0.54 and 0.45 min., respectively (figure 33). Buffer samples showed the least differences with an adapted – nonadapted difference of 0.26 min.

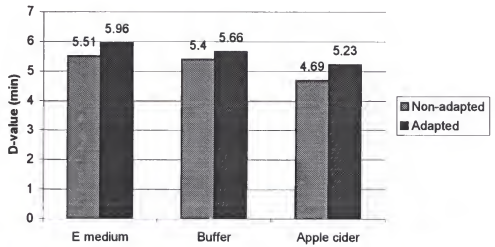

Figure 33. A comparison of acid-adapted and nonadapted D-values (RLU-derived count) for each matrix after thermal treatment.

Comparisons between acid-adapted and nonadapted cells after thermal treatments were fairly consistent. The line patterns portraying acid-adapted and nonadapted differences for buffer and apple cider were irregular. Ryu and Beuchat (1999) found that a consistent difference existed between acid-adapted and nonadapted cells with increasing time. The buffer and apple cider protective responses may be indicative of increased protection over time.

Strains A and C generally showed most cross protection according to D-value differences for all media. The control strain seemed moderately resistant in all matrices except in buffer. Strain B clearly showed the least cross-protection against thermal treatment when comparing D-values for adapted and nonadapted strains. Leyer and Johnson showed that acid-adapted S. typhimurium displayed higher resistance to 50°C than nonadapted cells. A study done comparing heat resistance (56°C) of acid-adapted to nonadapted E. coli O157:H7 cells by Rowe and Kirk (1999), showed that significant differences existed ($\alpha = 0.05$). Ryu and Beauchat (1999) found that significant heat resistance ($\alpha = 0.05$) was displayed when comparing acid-adapted to nonadapted E. coli O157:H7 cells at temperatures of 52 C and 54 C in apple cider. On the contrary, Buchanan and Edelson (1996) found that no heat resistance differences existed between acid-adapted and nonadapted E. coli O157:H7 in apple juice. The increased resistance may be a result of adaptive protein protection intended to prevent membrane rupture. Heyde and Portelier (1990) indicated that 6 rpoS dependent proteins were produced when E. coli were subjected to sudden declines of pH.

A matrix comparison of acid-adapted and nonadapted cells showed that E-medium seemed more favorable for cross-protection. As with benzoate/sorbate-treated samples, nutrients in E-medium may be used be the organism for protective responses

It is difficult to determine the treatment that induces the greatest cross protective response, as direct comparisons between the different D-value time units can not be considered valid. However, general trends may be important in efforts to understand protection by the cells. According to figure 34. the acid-adapted cells seemed to be most protective against benzoate/sorbate as acid-adapted and nonadapted differences were the greatest. Acid-adapted cells were more protected against heat than freeze-thaw in all media samples, as freeze-thaw acid-adapted showed slight differences from nonadapted cells.

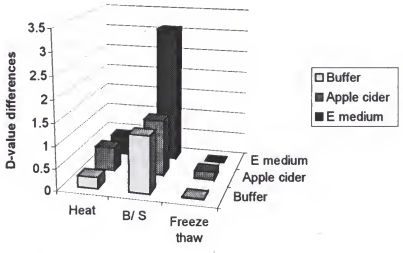

Figure 34. D-value differences (= acid-adapted – nonadapted) after heat (min.), benzoate/sorbate (days), and freeze – thaw (hours) for all matrices.

The exact mechanisms behind the cross protection exhibited can not be determined due to the scope of this study. However, by comparing findings from

studies that focus on cross protection at the molecular level with results from this study, conceivable mechanisms can be theorized. Protein synthesis, as an adaptive response for possible protection has been illustrated in several studies. The exact role of these proteins is not fully understood but it is fairly consensus that they contribute to general stress protection against all forms of environmental pressure. This is supported by evidence indicating that rpoS regulation is the main contributor to stress responses, though a few rpoS independent mechanisms have been observed. If rpoS offered primary protection, a strain with advanced protection system would show consistent cross protection against all forms of stress. This study agrees with rpos protection as indicated by increasing differences between adapted and nonadapted cells over time (Figure 19, 24, and 29). The differences may represent positive regulation of the rpoS operon by an autoinducer that becomes more abundant with increasing stress or time. The products of the operon are most likely utilized by the cell for protection, resulting in increasing differences in resistance between acid-adapted and nonadapted cells. However, data from this study also suggests that rpoS independent mechanisms may play a larger role in cross protection. This is evident as each strain shows varying protection against the different treatment.

SUMMARY AND CONCLUSION

In this study, three environmental E. coli O157:H7 strains and an E. coli control Ecor1were successfully electrotransformed with the lux plasmid pT7-5. The transformations did not alter wild type characteristics associated with the strains, except for the intended luciferase production. A linear regression analysis showed that all three strains showed excellent correlation R=0.90 between colony-forming units obtained from plate counts and relative light units obtained through luminometry. This correlation was observed for acid-adapted and nonadapted cells in E-medium, buffer, and apple cider. Statistical analyses (α =0.05) showed that the pT7-5 plasmid was stably maintained inside the cell after being subjected to maximum levels of treatment performed in this study.

Strain comparisons revealed that the strains variation existed after benzoate/sorbate, freeze-thaw, and thermal treatment. Benzoate and sorbate-treated acid-adapted E. coli O157:H7 cells showed between a 1.5 to 3-log reduction depending on the strain. E. coli O157:H7 cells in apple cider showed a 2.5-log decline after treatment with benzoate/sorbate. E. coli O157 showed greatest resistance to benzoate/sorbate treatment while the E. coli control was most sensitive. The acid-adapted E. coli O157 appeared to show highest survival in E-medium and lowest survival in buffer after benzoate/sorbate treatment.

E. coli O157:H7 showed a steady decline between 3 and 5.5-logs after freeze-thaw treatment. In apple cider, E. coli O157:H7 showed maximum decline of approximately 4-logs over 10 hours. E. coli O157:H7 strain mf6707.a seemed to show the greatest resistance, while strain mf1847 appeared to most sensitive. Maximal E. coli O157 declines occurred in apple cider and buffer after freeze-thaw treatment.

E. coli O157:H7 showed 3.5 to 7-log reduction in numbers after thermal treatment (54°C) depending on the strain. In apple cider, a 6-log drop was observed for E. coli O157:H7 cells. Heat resistant results were variable but strains 9124-1 and mf1847 appeared to be most resistant. E. coli O157:H7 cells in E-medium survived the best after thermal treatment.

An analysis for degree of sublethally injury was performed by comparing D-values using data from plate counts with RLU – derived counts. Raw data indicated that sublethal counts were nearly always greater than plate counts showing statistical significance (α =0.05) in several cases, especially for freeze-thaw-treated organisms. Strain 9124-1 showed the greatest difference between plate counts and RLU-derived counts indicating high sublethal injury. Sublethal injury was greatest in apple cider.

Cross protection was measured as the D-value difference between acid-adapted and nonadapted cells for benzoate/sorbate, freeze-thaw, and thermally-treated samples. Benzoate/sorbate-treated E. coli O157 showed significant (α =0.05) cross protection for all samples. Strains 9124-1 and mf6707.a showed the greatest degree of protection while the E. coli control showed the least in all matrices. E-medium allowed for the greatest cross protection.

Freeze-thawed samples did not show consistent cross protection. Strains mf6707.a and mf1847 appeared to display the highest degree of adaptive resistance, while protection for strain 9124-1 and control was minimal. The apple cider and buffer matrices seemed allow for greatest protection.

Thermally-treated samples showed significant (α =0.05) cross protection for all strains. Strains 9124-1 and mf1847 demonstrated the highest degree of protection, while strain mf6707.a showed the least. E-medium appeared to allow for greatest adaptive resistance. Generally, cross protection seemed to be most effective against benzoate/sorbate treatment, while protection against freeze-thaw seemed unclear.

Strain variation exhibited in this study is a good indication that survival studies including those involving cross protection should take into account the strains being used for the study. It would be highly inappropriate to make generalizations regarding the survival and cross protection of all E. coli O157:H7 based on a few strains. The significant (α =0.05) sublethal injury exhibited in this study, implied that survival studies that are conducted by merely determining colony-forming units may be inaccurate. Stressed cells that are unable to multiply should also be included in analyses as they can remain infective or may eventually recuperate to the vegetative state. Finally, the significant (α =0.05) cross protection encountered in this study is also very important from the public health standpoint. E. coli that are able to resist certain antimicrobial treatments will be more difficult to control in the food industry. Also, the low infective dose already associated with the organism may reduce further as ingested E. coli O157:H7 could resist stomach acids to colonize the lower intestine.

The use of a bioluminescent system appeared to be effective in measuring sublethal injury and cross protection in E. coli O157:H7. This approach to cross protection studies takes into account sublethal injured cells, that are almost certainly present after cells have been subjected to stress. Future survival studies involving apple cider and other matrices, should employ enumeration techniques to measure nonculturable bacteria as they have been shown to exist in significant quantities. The significant presence of sublethally injured cells may establish more accurate killing effects of some previously characterized preservation methods.

Also, future survival studies involving low pH matrices should consider using acid adapted organisms before subjecting samples to treatment, to take into account cross protection. Cross protection may eventually allow for an organism to survive harsher treatments such as currently implemented pasteurization techniques, leading to possible outbreaks. Therefore, it is important to continue studies that characterize the phenomenon to truly understand the survival potential of E. coli O157:H7. It is recommended that the juice and cider industry become updated to future findings regarding cross protection.

This study indicated mixed inferences regarding the control of E. coli O157:H7 in fresh juice and ciders. All three treatments appeared to lower E. coli O157:H7 counts and may be practical in intervention. However, the existence of cross protection against these treatments may be unfortunate to the struggling fresh juice and cider industry as provides further impetus to employ pasteurization regimens.

APPENDIX A.
COMPARISON OF WILD TYPE AND TRANSFORMANT API20E RESULTS

	Strain A		Strain B		Strain C		Control	
	Wildtype	Trans-formed	Wildtype	Trans-formed	Wildtype	Trans-formed	Wildtype	Trans-formed
O-nitrophenyl – ß-galactoside	+	+	+	+	+	+	+	+
Arginine dihydrolase	-	-	-	-	-	-	-	-
Lysine decarboxylase	+	+	+	+	+	+	+	+
Ornithine decarboxylase	+	+	+	+	-	-	+	+
Citrate utilization	-	-	-	-	-	-	-	-
Hydrogen sulfide production	-	-	-	-	-	-	-	-
Urea hydrolysis	-	-	-	-	-	-	-	-
Tryptophane deaminase	-	-	-	-	-	-	-	-
Indole production	+	+	+	+	+	+	+	+
Voges-Proskauer test	-	-	-	-	-	-	-	-
Gelatin hydrolysis	-	-	-	-	-	-	-	-
Glucose fermentation	+	+	+	+	+	+	+	+
Mannitol fermentation	+	+	+	+	+	+	+	+
Inositol fermentation	-	-	-	-	-	-	-	-
Sorbitol fermentation	-	-	-	-	-	-	-	-
Rhamnose fermentation	+	+	+	+	+	+	+	+
Saccharose fermentation	+	+	-	-	+	+	-	-
Melibiose fermentation	+	+	+	+	+	+	+	+
Amygdalin fermentation	-	-	-	-	-	-	-	-
Arabinose fermentation	+	+	+	+	+	+	+	+
Oxidase	-	-	-	-	-	-	-	-

APPENDIX B

SLOPE, INTERCEPTS, AND R-SQUARE VALUES AFTER LINEAR REGRESSION ANALYSIS CORRELATING CFU/ ML WITH RLU (X=CFU/ ML, Y=RLU)

Acid-adapted Cells

E. coli Strains	E medium			BPB			Apple Cider		
	Slope	y-intercept	R^2	Slope	y-intercept	R^2	Slope	y-intercept	R^2
A	1.0855	-1.243	0.9976	1.0731	-1.493	0.9973	1.0658	- 1.687	0.9967
B	1.0951	-1.170	0.9992	1.0876	-1.250	0.9989	1.1057	-1.503	0.9988
C	0.9605	-1.398	0.9863	0.9535	-1.605	0.9867	0.9420	1.754	0.9862
Control	1.1712	-0.981	0.9911	1.1628	-1.220	0.9922	1.1449	-1.200	0.9906

Nonadapted Cells

E. coli Strains	E medium			BPB			Apple Cider		
	Slope	y-intercept	R^2	Slope	y-intercept	R^2	Slope	y-intercept	R^2
A	1.2794	-0.819	0.9991	1.2470	-0.859	0.9988	1.2553	-1.108	0.9975
B	1.2138	-1.208	0.9934	1.1528	-1.081	0.9983	1.1662	-1.436	0.9996
C	1.2297	-0.962	0.9966	1.2179	-1.039	0.9978	1.1950	-1.240	0.9994
Control	1.2038	-0.207	0.9956	1.2126	-0.444	0.9956	1.2358	-1.623	0.9936

APPENDIX C

SLOPE, INTERCEPTS, AND R-SQUARE VALUES AFTER LINEAR REGRESSION ANALYSIS CORRELATING CFU/ ML WITH RLU (X=RLU, Y=CFU/ ML)

Acid-adapted Cells

E. coli Strains	E medium			BPB			Apple Cider		
	Slope	y-intercept	R^2	Slope	y-intercept	R^2	Slope	y-intercept	R^2
A	-0.9190	1.155	0.9976	-0.9293	1.401	0.9973	-0.9354	1.595	0.9980
B	-0.9125	1.071	0.9992	-0.9184	1.154	0.9989	-0.9032	1.354	0.9862
C	-1.0269	1.506	0.9863	-1.0348	1.731	0.9867	-1.0469	1.910	0.9906
Control	-0.8462	0.867	0.9911	-0.8533	1.074	0.9922	-0.8653	1.078	0.9989
Avg	-0.9718	0.996	0.9989	-0.9792	1.195	0.9985	-0.9873	1.334	

Nonadapted Cells

E. coli Strains	E medium			BPB			Apple Cider		
	Slope	y-intercept	R^2	Slope	y-intercept	R^2	Slope	y-intercept	R^2
A	-0.7809	0.643	0.9991	-0.8010	0.694	0.9988	-0.7946	0.895	0.9975
B	-0.8184	1.020	0.9934	-0.8660	0.944	0.9983	-0.8571	1.233	0.9996
C	-0.8105	0.798	0.9966	-0.8193	0.863	0.9978	-0.8363	1.040	0.9994
Control	-0.8271	0.194	0.9956	-0.8210	0.385	0.9956	-0.8041	1.337	0.9936
Avg	-0.8258	0.607	0.9993	-0.8269	0.714	0.9990	-0.8213	1.124	0.9991

APPENDIX D

T- TEST RESULTS (ASSUMING UNEQUAL VARIANCES) COMPARING PREDICTED AND OBSERVED (CFU/ ML VS. RLU) D-VALUES AFTER HEAT-TREATMENT (54°C) TO ASSESS TRANSFORMANT STABILITY

Heat Treatment in E-Medium

Acid-adapted

		Mean	Variance	df	t Stat	P(T<=t) one-tail	t Critical one-tail	P(T<=t) two-tail	t Critical two-tail
Strain A	Predicted	2.577	0.00103	4.000	1.109	0.165	2.132	0.329	2.776
	Observed	2.550	0.00070						
Strain B	Predicted	1.830	0.00070	3.000	-1.323	0.139	2.353	0.278	3.182
	Observed	1.853	0.00023						
Strain C	Predicted	3.503	0.00043	3.000	0.707	0.265	2.353	0.530	3.182
	Observed	3.487	0.00123						
Control	Predicted	0.917	0.00093	4.000	0.000	0.500	2.132	1.000	2.776
	Observed	0.917	0.00163						

Nonadapted

		Mean	Variance	df	t Stat	P(T<=t) one-tail	t Critical one-tail	P(T<=t) two-tail	t Critical two-tail
Strain A	Predicted	2.393	0.00013	3.000	-1.387	0.130	2.353	0.260	3.182
	Observed	2.410	0.00030						
Strain B	Predicted	1.060	0.00120	2.000	1.265	0.167	2.920	0.333	4.303
	Observed	1.033	0.00013						
Strain C	Predicted	2.647	0.00103	2.000	-1.200	0.176	2.920	0.353	4.303
	Observed	2.670	0.00010						
Control	Predicted	0.473	0.00123	4.000	-0.206	0.423	2.132	0.847	2.776
	Observed	0.480	0.00190						

Heat Treatment in Buffer

Acid-adapted

		Mean	Variance	df	t Stat	P(T<=t) one-tail	t Critical one-tail	P(T<=t) two-tail	t Critical two-tail
Strain A	Predicted	2.590	0.00070	4.000	0.316	0.384	2.132	0.768	2.776
	Observed	2.583	0.00063						
Strain B	Predicted	1.487	0.00123	4.000	0.849	0.222	2.132	0.444	2.776
	Observed	1.463	0.00103						
Strain C	Predicted	2.847	0.00103	2.000	-1.200	0.176	2.920	0.353	4.303
	Observed	2.870	0.00010						
Control	Predicted	1.070	0.00070	3.000	2.079	0.065	2.353	0.129	3.182
	Observed	1.033	0.00023						

Nonadapted

		Mean	Variance	df	t Stat	P(T<=t) one-tail	t Critical one-tail	P(T<=t) two-tail	t Critical two-tail
Strain A	Predicted	2.517	0.00103	3.000	-0.632	0.286	2.353	0.572	3.182
	Observed	2.530	0.00030						
Strain B	Predicted	1.087	0.00023	4.000	2.012	0.057	2.132	0.114	2.776
	Observed	1.057	0.00043						
Strain C	Predicted	1.407	0.00013	3.000	5.814	0.005	2.353	0.010	3.182
	Observed	1.363	0.00003						
Control	Predicted	0.477	0.00163	3.000	-0.254	0.408	2.353	0.816	3.182
	Observed	0.483	0.00043						

Heat Treatment in Apple Cider

Acid-adapted

		Mean	Variance	df	t Stat	P(T<=t) one-tail	t Critical one-tail	P(T<=t) two-tail	t Critical two-tail
Strain A	Predicted	2.763	0.04223	4.000	1.270	0.136	2.132	0.273	2.776
	Observed	2.513	0.07403						
Strain B	Predicted	0.773	0.00093	4.000	0.668	0.270	2.132	0.541	2.776
	Observed	0.757	0.00093						
Strain C	Predicted	2.067	0.00173	3.000	-0.875	0.223	2.353	0.446	3.182
	Observed	2.090	0.00040						
Control	Predicted	0.063	0.00003	2.000	1.151	0.184	2.920	0.369	4.303
	Observed	0.040	0.00120						

Nonadapted

		Mean	Variance	df	t Stat	P(T<=t) one-tail	t Critical one-tail	P(T<=t) two-tail	t Critical two-tail
Strain A	Predicted	1.523	0.01443	3.000	-0.853	0.228	2.353	0.456	3.182
	Observed	1.680	0.08680						
Strain B	Predicted	0.380	0.00030	4.000	1.964	0.061	2.132	0.121	2.776
	Observed	0.350	0.00040						
Strain C	Predicted	1.193	0.00013	3.000	-1.600	0.104	2.353	0.208	3.182
	Observed	1.220	0.00070						
Control	Predicted	0.033	0.00023	4.000	1.809	0.072	2.132	0.145	2.776
	Observed	0.013	0.00013						

APPENDIX E

T- TEST RESULTS (ASSUMING UNEQUAL VARIANCES) COMPARING PREDICTED AND OBSERVED (CFU/ ML VS. RLU) D-VALUES AFTER BENZOATE/SORBATE (0.1%) TREATMENT TO ASSESS TRANSFORMANT STABILITY

Benzoate and Sorbate Treatment in E-Medium

Acid-adapted

		Mean	Variance	df	t Stat	P(T<=t) one-tail	t Critical one-tail	P(T<=t) two-tail	t Critical two-tail
Strain A	Predicted	5.823	0.00023	2.000	-1.511	0.135	2.920	0.270	4.303
	Observed	5.867	0.00223						
Strain B	Predicted	6.387	0.00333	2.000	-0.896	0.233	2.920	0.465	4.303
	Observed	6.417	0.00003						
Strain C	Predicted	5.827	0.00023	3.000	-1.668	0.097	2.353	0.194	3.182
	Observed	5.853	0.00053						
Control	Predicted	4.457	0.00103	3.000	-0.610	0.292	2.353	0.585	3.182
	Observed	4.470	0.00040						

Nonadapted

		Mean	Variance	df	t Stat	P(T<=t) one-tail	t Critical one-tail	P(T<=t) two-tail	t Critical two-tail
Strain A	Predicted	5.623	0.00263	3.000	0.443	0.344	2.353	0.688	3.182
	Observed	5.597	0.00823						
Strain B	Predicted	6.403	0.00043	3.000	1.015	0.192	2.353	0.385	3.182
	Observed	6.370	0.00280						
Strain C	Predicted	4.697	0.00093	3.000	0.839	0.232	2.353	0.463	3.182
	Observed	4.670	0.00210						
Control	Predicted	3.510	0.00010	2.000	1.886	0.100	2.920	0.200	4.303
	Observed	3.473	0.00103						

Benzoate and Sorbate Treatment in Buffer

Acid-adapted

		Mean	Variance	df	t Stat	P(T<=t) one-tail	t Critical one-tail	P(T<=t) two-tail	t Critical two-tail
Strain A	Predicted	5.000	0.00120	2.000	-1.808	0.106	2.920	0.212	4.303
	Observed	5.037	0.00003						
Strain B	Predicted	5.620	0.00000	2.000	-7.000	0.010	2.920	0.020	4.303
	Observed	5.643	0.00003						
Strain C	Predicted	5.133	0.00063	3.000	-1.961	0.072	2.353	0.145	3.182
	Observed	5.167	0.00023						
Control	Predicted	4.217	0.00083	3.000	0.743	0.256	2.353	0.511	3.182
	Observed	4.203	0.00013						

Nonadapted

		Mean	Variance	df	t Stat	P(T<=t) one-tail	t Critical one-tail	P(T<=t) two-tail	t Critical two-tail
Strain A	Predicted	4.467	0.00223	4.000	0.431	0.344	2.132	0.689	2.776
	Observed	4.447	0.00423						
Strain B	Predicted	5.610	0.00130	4.000	1.359	0.123	2.132	0.246	2.776
	Observed	5.570	0.00130						
Strain C	Predicted	4.397	0.00023	3.000	0.603	0.295	2.353	0.589	3.182
	Observed	4.383	0.00123						
Control	Predicted	2.343	0.00043	3.000	1.273	0.146	2.353	0.293	3.182
	Observed	2.313	0.00123						

Benzoate and Sorbate Treatment in Buffer

Acid-adapted

		Mean	Variance	df	t Stat	P(T<=t) one-tail	t Critical one-tail	P(T<=t) two-tail	t Critical two-tail
Strain A	Predicted	4.887	0.00083	4.000	0.649	0.276	2.132	0.552	2.776
	Observed	4.873	0.00043						
Strain B	Predicted	5.877	0.00003	2.000	1.315	0.159	2.920	0.319	4.303
	Observed	5.850	0.00120						
Strain C	Predicted	4.543	0.00103	3.000	0.302	0.391	2.353	0.783	3.182
	Observed	4.537	0.00043						
Control	Predicted	4.343	0.00013	3.000	-1.387	0.130	2.353	0.260	3.182
	Observed	4.360	0.00030						

Nonadapted

		Mean	Variance	df	t Stat	P(T<=t) one-tail	t Critical one-tail	P(T<=t) two-tail	t Critical two-tail
Strain A	Predicted	3.733	0.00923	4.000	-0.138	0.449	2.132	0.897	2.776
	Observed	3.743	0.00663						
Strain B	Predicted	5.030	0.00790	4.000	0.145	0.446	2.132	0.892	2.776
	Observed	5.020	0.00640						
Strain C	Predicted	3.900	0.00090	4.000	0.158	0.441	2.132	0.882	2.776
	Observed	3.897	0.00043						
Control	Predicted	2.387	0.00173	3.000	-0.125	0.454	2.353	0.908	3.182
	Observed	2.390	0.00040						

APPENDIX F

T- TEST RESULTS (ASSUMING UNEQUAL VARIANCES) COMPARING PREDICTED AND OBSERVED (CFU/ ML VS. RLU) D-VALUES AFTER FREEZE-THAW TREATMENT TO ASSESS TRANSFORMANT STABILITY

Freeze-thaw in E -Medium

Acid-adapted

		Mean	Variance	df	t Stat	P(T<=t) one-tail	t Critical one-tail	P(T<=t) two-tail	t Critical two-tail
Strain A	Predicted	2.823	0.10943	3.000	0.901	0.217	2.353	0.434	3.182
	Observed	2.640	0.01470						
Strain B	Predicted	2.030	0.00390	2.000	2.065	0.087	2.920	0.175	4.303
	Observed	1.953	0.00023						
Strain C	Predicted	3.613	0.00003	2.000	1.265	0.167	2.920	0.333	4.303
	Observed	3.600	0.00030						
Control	Predicted	2.457	0.00063	3.000	2.345	0.050	2.353	0.101	3.182
	Observed	2.420	0.00010						

Nonadapted

		Mean	Variance	df	t Stat	P(T<=t) one-tail	t Critical one-tail	P(T<=t) two-tail	t Critical two-tail
Strain A	Predicted	2.593	0.00053	4.000	1.400	0.117	2.132	0.234	2.776
	Observed	2.570	0.00030						
Strain B	Predicted	1.793	0.00053	3.000	2.294	0.053	2.353	0.106	3.182
	Observed	1.760	0.00010						
Strain C	Predicted	2.580	0.10830	2.000	-1.159	0.183	2.920	0.366	4.303
	Observed	2.807	0.00653						
Control	Predicted	2.860	0.05470	4.000	2.623	0.029	2.132	0.059	2.776
	Observed	2.347	0.06023						

Freeze-thaw in Buffer

Acid-adapted

		Mean	Variance	df	t Stat	P(T<=t) one-tail	t Critical one-tail	P(T<=t) two-tail	t Critical two-tail
Strain A	Predicted	2.237	0.00013	2.000	1.265	0.167	2.920	0.333	4.303
	Observed	2.210	0.00120						
Strain B	Predicted	1.453	0.00163	2.000	0.707	0.276	2.920	0.553	4.303
	Observed	1.437	0.00003						
Strain C	Predicted	2.893	0.00023	3.000	-2.530	0.043	2.353	0.085	3.182
	Observed	2.920	0.00010						
Control	Predicted	2.407	0.02893	4.000	1.662	0.086	2.132	0.172	2.776
	Observed	2.183	0.02523						

Nonadapted

		Mean	Variance	df	t Stat	P(T<=t) one-tail	t Critical one-tail	P(T<=t) two-tail	t Critical two-tail
Strain A	Predicted	2.287	0.00013	2.000	0.791	0.256	2.920	0.512	4.303
	Observed	2.270	0.00120						
Strain B	Predicted	1.327	0.00093	3.000	0.707	0.265	2.353	0.530	3.182
	Observed	1.313	0.00013						
Strain C	Predicted	2.323	0.00023	4.000	2.012	0.057	2.132	0.114	2.776
	Observed	2.293	0.00043						
Control	Predicted	1.970	0.00070	2.000	1.492	0.137	2.920	0.274	4.303
	Observed	1.947	0.00003						

Freeze-thaw in Buffer

Acid-adapted

		Mean	Variance	df	t Stat	P(T<=t) one-tail	t Critical one-tail	P(T<=t) two-tail	t Critical two-tail
Strain A	Predicted	1.760	0.00090	4.000	0.539	0.309	2.132	0.618	2.776
	Observed	1.747	0.00093						
Strain B	Predicted	1.483	0.00003	2.000	1.151	0.184	2.920	0.369	4.303
	Observed	1.460	0.00120						
Strain C	Predicted	3.667	0.00093	2.000	1.984	0.093	2.920	0.186	4.303
	Observed	3.333	0.08373						
Control	Predicted	2.123	0.00063	4.000	-0.791	0.237	2.132	0.473	2.776
	Observed	2.140	0.00070						

Nonadapted

		Mean	Variance	df	t Stat	P(T<=t) one-tail	t Critical one-tail	P(T<=t) two-tail	t Critical two-tail
Strain A	Predicted	1.680	0.00120	2.000	1.151	0.184	2.920	0.369	4.303
	Observed	1.657	0.00003						
Strain B	Predicted	1.240	0.00070	3.000	1.643	0.099	2.353	0.199	3.182
	Observed	1.210	0.00030						
Strain C	Predicted	2.583	0.00083	3.000	-1.061	0.183	2.353	0.367	3.182
	Observed	2.603	0.00023						
Control	Predicted	1.490	0.00010	2.000	1.715	0.114	2.920	0.228	4.303
	Observed	1.457	0.00103						

APPENDIX G
D-VALUES USING DATA FROM PLATE COUNTS (CFU) AND RLU-DERIVED COUNTS (DCFU) FOR ACID-ADAPTED AND NONADAPTED CELLS AFTER HEAT TREATMENT (54°C) IN ALL MATRICES

Heat-Treated Acid-Adapted Cells

	E. coli Strains	E medium				BPB				Apple Cider			
		Slope	y-intercept	R^2	D value	Slope	y-intercept	R^2	D value	Slope	y-intercept	R^2	D value
CFU	A	-0.1666	7.920	0.9736	6.00	-0.1649	7.808	0.9822	6.06	-0.1667	7.539	0.9952	6.00
	B	-0.2091	7.632	0.9703	4.78	-0.2102	7.422	0.9803	4.76	-0.2242	7.040	0.9854	4.46
	C	-0.1512	8.061	0.9534	6.61	-0.1623	8.129	0.9655	6.16	-0.1862	7.493	0.9803	5.37
	Control	-0.2349	6.941	0.9280	4.26	-0.1846	5.837	0.8408	5.42	-0.2491	6.339	0.914	4.01
	Average	-0.1756	7.871	0.9923	5.69	-0.1791	7.786	0.9932	5.58	-0.1924	7.358	0.9933	5.20
dCFU	A	-0.1551	8.163	0.9852	6.45	-0.1623	8.090	0.9863	6.16	-0.1911	7.799	0.9906	5.23
	B	-0.2036	7.806	0.9700	4.91	-0.2093	7.778	0.9839	4.78	-0.2298	7.334	0.9891	4.35
	C	-0.1446	8.200	0.9670	6.92	-0.1586	8.389	0.9605	6.31	-0.1825	7.628	0.9784	5.48
	Control	-0.2363	7.119	0.9256	4.23	-0.185	6.229	0.8437	5.41	-0.2548	6.619	0.9294	3.92
	Average	-0.1678	8.056	0.9869	5.96	-0.1767	8.086	0.9921	5.66	-0.2011	7.587	0.9936	4.97

Heat-Treated Nonadapted Cells

	E. coli Strains	E medium				BPB				Apple Cider			
		Slope	y-intercept	R^2	D value	Slope	y-intercept	R^2	D value	Slope	y-intercept	R^2	D value
CFU	A	-0.1713	7.886	0.9849	5.84	-0.1718	7.900	0.9862	5.82	-0.1861	7.391	0.9947	5.37
	B	-0.2204	7.115	0.9685	4.54	-0.2216	7.151	0.9688	4.51	-0.236	7.014	0.9734	4.24
	C	-0.1614	7.928	0.9728	6.20	-0.1647	7.832	0.9719	6.07	-0.223	7.842	0.9909	4.48
	Control	-0.2406	6.841	0.9312	4.16	-0.2375	6.801	0.932	4.21	-0.2648	6.586	0.883	3.78
	Average	-0.1843	7.643	0.9943	5.43	-0.186	7.628	0.9942	5.38	-0.215	7.416	0.9968	4.65
dCFU	A	-0.1604	8.021	0.962	6.23	-0.1728	8.062	0.9862	5.79	-0.1944	7.783	0.9938	5.14
	B	-0.2029	7.370	0.9719	4.93	-0.2193	7.452	0.972	4.56	-0.2324	7.453	0.9709	4.30
	C	-0.1809	7.987	0.9908	5.53	-0.2059	7.638	0.9944	4.86	-0.2209	8.231	0.9865	4.53
	Control	-0.2381	7.076	0.9355	4.20	-0.2367	7.092	0.9358	4.22	-0.2714	7.110	0.9217	3.68
	Average	-0.1814	7.793	0.9954	5.51	-0.1993	7.717	0.9985	5.02	-0.2159	7.822	0.997	4.63

APPENDIX H
D-VALUES USING DATA FROM PLATE COUNTS (CFU) AND RLU-DERIVED COUNTS (DCFU) FOR ACID-ADAPTED AND NONADAPTED CELLS AFTER BENZOATE/SORBATE (0.1 %) TREATMENT IN ALL MATRICES

Heat-Treated Acid-Adapted Cells

	E. coli Strains	E medium				BPB				Apple Cider			
		Slope	y-intercept	R^2	D value	Slope	y-intercept	R^2	D value	Slope	y-intercept	R^2	D value
CFU	A	-0.1571	7.363	0.9740	6.37	-0.2168	7.159	0.9926	4.61	-0.2250	7.032	0.9699	4.44
	B	-0.1024	7.293	0.9859	9.77	-0.1477	7.465	0.9796	6.77	-0.1687	7.279	0.9761	5.93
	C	-0.1362	7.133	0.9470	7.34	-0.224	7.146	0.9832	4.46	-0.2516	7.152	0.9894	3.97
	Control	-0.3027	7.315	0.9513	3.30	-0.3095	7.124	0.9476	3.23	-0.3076	6.885	0.8996	3.25
	Average	-0.1319	7.395	0.9756	7.58	-0.2046	7.503	0.9871	4.89	-0.2976	7.239	0.9743	3.36
dCFU	A	-0.1552	7.443	0.9762	6.44	-0.2164	7.347	0.9889	4.62	-0.2231	7.250	0.9614	4.48
	B	-0.0995	7.543	0.9744	10.05	-0.1683	7.333	0.9779	5.94	-0.1714	7.395	0.9798	5.83
	C	-0.1271	7.276	0.9365	7.87	-0.2287	7.365	0.9790	4.37	-0.2507	7.443	0.9948	3.99
	Control	-0.3027	7.056	0.9548	3.30	-0.3323	7.261	0.9676	3.01	-0.3149	7.109	0.9034	3.18
	Average	-0.1272	7.548	0.9700	7.86	-0.1960	7.516	0.9893	5.10	-0.3005	7.146	0.9773	3.33

Heat-Treated Nonadapted Cells

	E. coli Strains	E medium				BPB				Apple Cider			
		Slope	y-intercept	R^2	D value	Slope	y-intercept	R^2	D value	Slope	y-intercept	R^2	D value
CFU	A	-0.2388	7.423	0.9599	4.19	-0.3115	7.591	0.9951	3.21	-0.3128	7.207	0.9742	3.20
	B	-0.1257	7.567	0.9889	7.96	-0.1855	7.391	0.9760	5.39	-0.2311	7.410	0.9701	4.33
	C	-0.2714	7.496	0.9797	3.68	-0.2927	7.291	0.9932	3.42	-0.3488	7.410	0.9722	2.87
	Control	-0.3506	7.705	0.9549	2.85	-0.4820	7.535	0.9856	2.07	-0.4958	7.303	0.9788	2.02
	Average	-0.212	7.495	0.9906	4.72	-0.2632	7.424	0.9968	3.80	-0.3041	7.410	0.9795	3.29
dCFU	A	-0.2415	7.778	0.9851	4.14	-0.3175	7.394	0.9881	3.15	-0.3253	7.003	0.9846	3.07
	B	-0.1237	7.665	0.9801	8.08	-0.1833	7.519	0.9865	5.46	-0.2381	7.212	0.9696	4.20
	C	-0.2682	7.770	0.9758	3.73	-0.2919	7.457	0.9943	3.43	-0.3381	7.224	0.9612	2.96
	Control	-0.4386	7.841	0.9874	2.28	-0.5066	7.377	0.9972	1.97	-0.4652	7.320	0.9967	2.15
	Average	-0.2111	7.738	0.9945	4.74	-0.2642	7.457	0.9936	3.79	-0.2940	7.574	0.9714	3.40

APPENDIX I

D-VALUES USING DATA FROM PLATE COUNTS (CFU) AND RLU-DERIVED COUNTS (DCFU) FOR ACID-ADAPTED AND NONADAPTED CELLS AFTER FREEZE-THAW TREATMENT IN ALL MATRICES

Freeze-Thaw-Treated Acid-Adapted Cells

	E. coli Strains	E medium				BPB				Apple Cider			
		Slope	y-intercept	R^2	D value	Slope	y-intercept	R^2	D value	Slope	y-intercept	R^2	D value
CFU	A	-0.4256	6.861	0.9834	2.35	-0.5062	7.211	0.9959	1.98	-0.5206	6.951	0.9954	1.92
	B	-0.4948	6.613	0.9848	2.02	-0.5505	6.723	0.9838	1.82	-0.5370	6.432	0.9631	1.86
	C	-0.3535	7.491	0.9559	2.83	-0.4281	7.584	0.9851	2.34	-0.4256	7.396	0.9906	2.35
	Control	-0.4306	6.718	0.9838	2.32	-0.4967	6.883	0.9839	2.01	-0.4783	6.709	0.9738	2.09
	Average	-0.4247	6.989	0.9906	2.35	-0.4949	7.173	0.9957	2.02	-0.4944	6.926	0.9948	2.02
dCFU	A	-0.4044	7.145	0.9854	2.47	-0.4777	7.384	0.9962	2.09	-0.4715	7.046	0.9918	2.12
	B	-0.4654	6.792	0.9780	2.15	-0.5127	6.906	0.9811	1.95	-0.4952	6.556	0.9604	2.02
	C	-0.3425	7.589	0.9554	2.92	-0.3997	7.825	0.9944	2.50	-0.3935	7.511	0.9831	2.54
	Control	-0.4295	6.888	0.9838	2.33	-0.4632	7.059	0.9860	2.16	-0.4337	7.176	0.9888	2.31
	Average	-0.4041	7.175	0.9909	2.47	-0.4634	7.372	0.9967	2.16	-0.4534	7.037	0.9936	2.21

Freeze-Thaw-Treated Acid-Adapted Cells

	E. coli Strains	E medium				BPB				Apple Cider			
		Slope	y-intercept	R^2	D value	Slope	y-intercept	R^2	D value	Slope	y-intercept	R^2	D value
CFU	A	-0.4245	7.101	0.9721	2.36	-0.4880	7.026	0.9895	2.05	-0.5301	6.875	0.9887	1.89
	B	-0.5084	6.551	0.9826	1.97	-0.5507	6.578	0.9769	1.82	-0.5575	6.326	0.9592	1.79
	C	-0.4088	7.340	0.9663	2.45	-0.4758	7.462	0.9856	2.10	-0.4630	7.333	0.9958	2.16
	Control	-0.4555	6.749	0.9883	2.20	-0.4964	6.828	0.9826	2.01	-0.5370	6.624	0.9765	1.86
	Average	-0.4472	6.997	0.990	2.24	-0.5048	7.022	0.9930	1.98	-0.5169	6.845	0.9890	1.93
dCFU	A	-0.3914	7.234	0.9780	2.55	-0.4589	7.322	0.9905	2.18	-0.4756	6.949	0.9826	2.10
	B	-0.5076	6.757	0.9850	1.97	-0.5215	6.760	0.9775	1.92	-0.5497	6.596	0.9621	1.82
	C	-0.3710	7.552	0.9542	2.70	-0.4209	7.661	0.9910	2.38	-0.4597	7.473	0.9952	2.18
	Control	-0.4295	6.940	0.9869	2.33	-0.4472	6.981	0.9712	2.24	-0.4993	6.739	0.9513	2.00
	Average	-0.4233	7.181	0.9894	2.36	-0.4671	7.247	0.9940	2.14	-0.4950	7.006	0.9868	2.02

APPENDIX J
T-TEST RESULTS ASSESSING SUBLEITHAL INJURY BY COMPARING PLATE COUNTS (CFU) WITH RLU-DERIVED COUNTS (DCFU) AFTER HEAT TREATMENT (54°C)

E-Medium

Acid-dapted

		Mean	Variance	df	t Stat	P(T<=t) one-tail	t Critical one-tail	P(T<=t) two-tail	t Critical two-tail
Strain A	dCFU	6.447	0.00143	4	15.076	0.000	2.132	0.000*	2.776
	CFU	6.000	0.00120						
Strain B	dCFU	4.913	0.00663	3	2.500	0.044*	2.353	0.088	3.182
	CFU	4.780	0.00190						
Strain C	dCFU	6.917	0.00043	2	8.239	0.007	2.920	0.014*	4.303
	CFU	6.613	0.00363						
Control	dCFU	4.260	0.00040	3	1.162	0.165	2.353	0.329	3.182
	CFU	4.230	0.00160						
Avg	dCFU	5.957	0.00463	4	5.388	0.003*	2.132	0.006*	2.776
	CFU	5.693	0.00253						

Nonadapted

		Mean	Variance	df	t Stat	P(T<=t) one-tail	t Critical one-tail	P(T<=t) two-tail	t Critical two-tail
Strain A	dCFU	6.237	0.00573	3	8.485	0.002	2.353	0.003*	3.182
	CFU	5.837	0.00093						
Strain B	dCFU	4.927	0.00093	3	8.845	0.002	2.353	0.003*	3.182
	CFU	4.540	0.00480						
Strain C	dCFU	6.197	0.00013	2	24.931	0.001	2.920	0.002*	4.303
	CFU	5.527	0.00203						
Control	dCFU	4.200	0.00010	2	0.944	0.222	2.920	0.445	4.303
	CFU	4.153	0.00723						
Avg	dCFU	5.510	0.00070	3	2.015	0.069	2.353	0.137	3.182
	CFU	5.427	0.00443						

Buffer

Acid-adapted

		Mean	Variance	df	t Stat	P(T<=t) one-tail	t Critical one-tail	P(T<=t) two-tail	t Critical two-tail
Strain A	dCFU	6.163	0.00093	4	4.376	0.006	2.132	0.012*	2.776
	CFU	6.063	0.00063						
Strain B	dCFU	4.780	0.00520	4	0.344	0.374	2.132	0.748	2.776
	CFU	4.757	0.00863						
Strain C	dCFU	6.303	0.00253	4	2.899	0.022	2.132	0.044*	2.776
	CFU	6.160	0.00480						
Control	dCFU	5.407	0.00503	2	-0.316	0.391	2.920	0.782	4.303
	CFU	5.420	0.00030						
Avg	dCFU	5.657	0.00093	2	4.085	0.028	2.920	0.055	4.303
	CFU	5.583	0.00003						

Nonadapted

		Mean	Variance	df	t Stat	P(T<=t) one-tail	t Critical one-tail	P(T<=t) two-tail	t Critical two-tail
Strain A	dCFU	5.820	0.00070	3	2.000	0.070	2.353	0.139	3.182
	CFU	5.787	0.00013						
Strain B	dCFU	4.563	0.00823	3	0.868	0.225	2.353	0.449	3.182
	CFU	4.510	0.00310						
Strain C	dCFU	6.070	0.01710	2	15.521	0.002	2.920	0.004*	4.303
	CFU	4.857	0.00123						
Control	dCFU	4.223	0.00043	2	0.258	0.410	2.920	0.821	4.303
	CFU	4.210	0.00760						
Avg	dCFU	5.397	0.00053	4	18.091	0.000	2.132	0.000*	2.776
	CFU	4.997	0.00093						

Apple Cider

Acid-adapted

		Mean	Variance	df	t Stat	P(T<=t) one-tail	t Critical one-tail	P(T<=t) two-tail	t Critical two-tail
Strain A	dCFU	5.997	0.00343	4	14.656	0.000	2.132	0.000*	2.776
	CFU	5.237	0.00463						
Strain B	dCFU	4.460	0.00910	3	1.713	0.093	2.353	0.185	3.182
	CFU	4.353	0.00253						
Strain C	dCFU	5.480	0.01510	2	1.530	0.133	2.920	0.266	4.303
	CFU	5.370	0.00040						
Control	dCFU	4.013	0.00343	4	1.867	0.068	2.132	0.135	2.776
	CFU	3.927	0.00303						
Avg	dCFU	4.013	0.00343	4	1.867	0.068	2.132	0.135	2.776
	CFU	3.927	0.00303						

Nonadapted

		Mean	Variance	df	t Stat	P(T<=t) one-tail	t Critical one-tail	P(T<=t) two-tail	t Critical two-tail
Strain A	dCFU	5.373	0.01003	3	3.635	0.018	2.353	0.036*	3.182
	CFU	5.147	0.00163						
Strain B	dCFU	4.303	0.00163	3	1.248	0.150	2.353	0.301	3.182
	CFU	4.237	0.00693						
Strain C	dCFU	4.530	0.00310	2	1.400	0.148	2.920	0.296	4.303
	CFU	4.483	0.00023						
Control	dCFU	3.683	0.00543	3	-2.104	0.063	2.353	0.126	3.182
	CFU	3.780	0.00090						
Avg	dCFU	4.693	0.00963	2	1.744	0.112	2.920	0.223	4.303
	CFU	4.593	0.00023						

* These values are statistically significant ($P < 0.05$)

APPENDIX K
T-TEST RESULTS ASSESSING SUBLETHAL INJURY BY COMPARING PLATE COUNTS (CFU) WITH RLU-DERIVED COUNTS (DCFU) AFTER FREEZE-THAW TREATMENT

E-Medium

Acid-adapted

		Mean	Variance	df	t Stat	P(T<=t) one-tail	t Critical one-tail	P(T<=t) two-tail	t Critical two-tail
Strain A	dCFU	2.473	0.01803	2	1.536	0.132	2.920	0.264	4.303
	CFU	2.350	0.00130						
Strain B	dCFU	2.150	0.00360	3	3.223	0.024	2.353	0.048*	3.182
	CFU	2.023	0.00103						
Strain C	dCFU	2.917	0.00053	2	1.045	0.203	2.920	0.406	4.303
	CFU	2.830	0.02010						
Control	dCFU	2.330	0.00390	2	0.175	0.438	2.920	0.877	4.303
	CFU	2.323	0.00043						
Avg	dCFU	2.477	0.01083	2	2.049	0.088	2.920	0.177	4.303
	CFU	2.353	0.00003						

Nonadapted

		Mean	Variance	df	t Stat	P(T<=t) one-tail	t Critical one-tail	P(T<=t) two-tail	t Critical two-tail
Strain A	dCFU	2.553	0.00023	2	3.128	0.044*	2.920	0.089	4.303
	CFU	2.353	0.01203						
Strain B	dCFU	1.970	0.00970	3	0.050	0.481	2.353	0.963	3.182
	CFU	1.967	0.00343						
Strain C	dCFU	2.693	0.00463	4	3.668	0.011	2.132	0.021*	2.776
	CFU	2.447	0.00893						
Control	dCFU	2.330	0.00390	3	3.322	0.022	2.353	0.045*	3.182
	CFU	2.197	0.00093						
Avg	dCFU	2.473	0.00043	3	3.337	0.022	2.353	0.044*	3.182
	CFU	2.357	0.00323						

Buffer

Acid-adapted

		Mean	Variance	df	t Stat	P(T<=t) one-tail	t Critical one-tail	P(T<=t) two-tail	t Critical two-tail
Strain A	dCFU	2.093	0.00173	4	2.733	0.026	2.132	0.052	2.776
	CFU	1.977	0.00373						
Strain B	dCFU	1.950	0.00130	3	3.430	0.021	2.353	0.042*	3.182
	CFU	1.817	0.00323						
Strain C	dCFU	2.500	0.00280	2	5.315	0.017	2.920	0.034*	4.303
	CFU	2.337	0.00003						
Control	dCFU	2.157	0.00243	3	4.216	0.012	2.353	0.024*	3.182
	CFU	2.013	0.00103						
Avg	dCFU	2.157	0.00043	4	7.071	0.001	2.132	0.002*	2.776
	CFU	2.023	0.00063						

Nonadapted

		Mean	Variance	df	t Stat	P(T<=t) one-tail	t Critical one-tail	P(T<=t) two-tail	t Critical two-tail
Strain A	dCFU	2.180	0.00090	2	7.120	0.010	2.920	0.019*	4.303
	CFU	2.050	0.00010						
Strain B	dCFU	1.917	0.00443	3	1.071	0.181	2.353	0.363	3.182
	CFU	1.817	0.02173						
Strain C	dCFU	2.377	0.00723	4	4.020	0.008	2.132	0.016*	2.776
	CFU	2.103	0.00663						
Control	dCFU	2.240	0.01330	2	3.279	0.041*	2.920	0.082	4.303
	CFU	2.013	0.00103						
Avg	dCFU	2.143	0.00543	2	3.736	0.032*	2.920	0.065	4.303
	CFU	1.980	0.00030						

Apple Cider

Acid-adapted

		Mean	Variance	df	t Stat	P(T<=t) one-tail	t Critical one-tail	P(T<=t) two-tail	t Critical two-tail
Strain A	dCFU	2.120	0.00280	3	5.855	0.005	2.353	0.010*	3.182
	CFU	1.920	0.00070						
Strain B	dCFU	2.017	0.00703	2	3.147	0.044*	2.920	0.088	4.303
	CFU	1.860	0.00040						
Strain C	dCFU	2.543	0.00043	2	4.081	0.028*	2.920	0.055	4.303
	CFU	2.350	0.00630						
Control	dCFU	2.307	0.00343	4	4.643	0.005	2.132	0.010*	2.776
	CFU	2.090	0.00310						
Avg	dCFU	2.207	0.00063	4	8.922	0.000	2.132	0.001*	2.776
	CFU	2.023	0.00063						

Nonadapted

		Mean	Variance	df	t Stat	P(T<=t) one-tail	t Critical one-tail	P(T<=t) two-tail	t Critical two-tail
Strain A	dCFU	2.103	0.00443	4	4.598	0.005	2.132	0.010*	2.776
	CFU	1.883	0.00243						
Strain B	dCFU	1.820	0.00210	3	0.418	0.352	2.353	0.704	3.182
	CFU	1.797	0.00723						
Strain C	dCFU	2.173	0.00063	2	0.127	0.455	2.920	0.911	4.303
	CFU	2.163	0.01803						
Control	dCFU	2.003	0.00043	2	3.542	0.036*	2.920	0.071	4.303
	CFU	1.867	0.00403						
Avg	dCFU	2.020	0.00970	3	1.423	0.125	2.353	0.250	3.182
	CFU	1.933	0.00143						

* These values are statistically significant ($P < 0.05$)

APPENDIX L
T-TEST RESULTS ASSESSING SUBLEITHAL INJURY BY COMPARING PLATE COUNTS (CFU) WITH RLU-DERIVED COUNTS (DCFU) AFTER BENZOATE/SORBATE (0.1%) TREATMENT

E-Medium

Acid-adapted

		Mean	Variance	df	t Stat	P(T<=t) one-tail	t Critical one-tail	P(T<=t) two-tail	t Critical two-tail
Strain A	dCFU	6.443	0.00023	2	1.524	0.133	2.920	0.267	4.303
	CFU	6.363	0.00803						
Strain B	dCFU	10.047	0.00263	4	7.426	0.001	2.132	0.002*	2.776
	CFU	9.763	0.00173						
Strain C	dCFU	7.867	0.00163	3	18.885	0.000	2.353	0.000*	3.182
	CFU	7.340	0.00070						
Control	dCFU	3.303	0.00163	4	0.000	0.500	2.132	1.000	2.776
	CFU	3.303	0.00333						
Avg	dCFU	7.863	0.00043	2	6.952	0.010	2.920	0.020*	4.303
	CFU	7.583	0.00443						

Nonadapted

		Mean	Variance	df	t Stat	P(T<=t) one-tail	t Critical one-tail	P(T<=t) two-tail	t Critical two-tail
Strain A	dCFU	4.187	0.00083	3	1.289	0.144	2.353	0.288	3.182
	CFU	4.140	0.00310						
Strain B	dCFU	8.087	0.00523	4	1.776	0.075	2.132	0.150	2.776
	CFU	7.957	0.01083						
Strain C	dCFU	3.730	0.00070	3	1.436	0.123	2.353	0.247	3.182
	CFU	3.687	0.00203						
Control	dCFU	2.850	0.00670	3	10.129	0.001	2.353	0.002*	3.182
	CFU	2.280	0.00280						
Avg	dCFU	4.757	0.00443	2	1.412	0.147	2.920	0.294	4.303
	CFU	4.700	0.00040						

Buffer

Acid-adapted

		Mean	Variance	df	t Stat	P(T<=t) one-tail	t Critical one-tail	P(T<=t) two-tail	t Critical two-tail
Strain A	dCFU	4.620	0.00070	2	0.120	0.458	2.920	0.916	4.303
	CFU	4.613	0.00863						
Strain B	dCFU	6.770	0.00190	4	26.437	0.000	2.132	0.000	2.776
	CFU	5.943	0.00103						
Strain C	dCFU	4.463	0.00823	2	1.694	0.116	2.920	0.232	4.303
	CFU	4.373	0.00023						
Control	dCFU	3.230	0.00040	2	2.848	0.052	2.920	0.104	4.303
	CFU	3.010	0.01750						
Avg	dCFU	5.103	0.01373	2	3.076	0.046*	2.920	0.091	4.303
	CFU	4.890	0.00070						

Nonadapted

		Mean	Variance	df	t Stat	P(T<=t) one-tail	t Critical one-tail	P(T<=t) two-tail	t Critical two-tail
Strain A	dCFU	3.210	0.00130	4	2.078	0.053	2.132	0.106	2.776
	CFU	3.150	0.00120						
Strain B	dCFU	5.457	0.00123	4	2.049	0.055	2.132	0.110	2.776
	CFU	5.393	0.00163						
Strain C	dCFU	3.427	0.00003	3	1.061	0.183	2.353	0.367	3.182
	CFU	3.417	0.00023						
Control	dCFU	2.077	0.00303	3	1.100	0.176	2.353	0.352	3.182
	CFU	1.973	0.02343						
Avg	dCFU	3.813	0.01203	3	0.636	0.285	2.353	0.570	3.182
	CFU	3.770	0.00190						

Apple Cider

Acid-adapted

		Mean	Variance	df	t Stat	P(T<=t) one-tail	t Critical one-tail	P(T<=t) two-tail	t Critical two-tail
Strain A	dCFU	4.480	0.00310	2	1.000	0.211	2.920	0.423	4.303
	CFU	4.447	0.00023						
Strain B	dCFU	5.927	0.00163	2	1.222	0.173	2.920	0.346	4.303
	CFU	5.837	0.01463						
Strain C	dCFU	3.250	0.00070	2	1.584	0.127	2.920	0.254	4.303
	CFU	3.177	0.00573						
Control	dCFU	3.250	0.00070	2	1.584	0.127	2.920	0.254	4.303
	CFU	3.177	0.00573						
Avg	dCFU	4.670	0.00190	3	0.765	0.250	2.353	0.500	3.182
	CFU	4.630	0.00630						

Nonadapted

		Mean	Variance	df	t Stat	P(T<=t) one-tail	t Critical one-tail	P(T<=t) two-tail	t Critical two-tail
Strain A	dCFU	3.197	0.00013	3	7.600	0.002	2.353	0.005*	3.182
	CFU	3.070	0.00070						
Strain B	dCFU	4.330	0.00030	3	5.898	0.005	2.353	0.010*	3.182
	CFU	4.197	0.00123						
Strain C	dCFU	2.957	0.00053	2	1.841	0.103	2.920	0.207	4.303
	CFU	2.867	0.00663						
Control	dCFU	2.150	0.00030	2	2.272	0.076	2.920	0.151	4.303
	CFU	2.017	0.01003						
Avg	dCFU	3.400	0.00370	3	1.363	0.133	2.353	0.266	3.182
	CFU	3.287	0.01703						

* These values are statistically significant ($P < 0.05$)

APPENDIX M

T-TEST RESULTS ASSESSING CROSS PROTECTION BY COMPARING ACID-ADAPTED WITH NONADAPTED AFTER HEAT TREATMENT (54°C) USING RLU-DERIVED COUNT VALUES

E-Medium

		Mean	Variance	df	t Stat	P(T<=t) one-tail	t Critical one-tail	P(T<=t) two-tail	t Critical two-tail
Strain A	adapted	6.447	0.00143	3.000	4.297	0.012	2.353	0.023*	3.182
	nonadapted	6.237	0.00573						
Strain B	adapted	4.913	0.00663	3.000	-0.265	0.404	2.353	0.808	3.182
	nonadapted	4.927	0.00093						
Strain C	adapted	6.917	0.00043	3.000	52.388	0.000	2.353	0.000*	3.182
	nonadapted	6.197	0.00013						
Control	adapted	4.260	0.00040	3.000	4.648	0.009	2.353	0.019*	3.182
	nonadapted	4.200	0.00010						
Average	adapted	5.957	0.00463	3.000	10.594	0.001	2.353	0.002*	3.182
	nonadapted	5.510	0.00070						

Buffer

		Mean	Variance	df	t Stat	P(T<=t) one-tail	t Critical one-tail	P(T<=t) two-tail	t Critical two-tail
Strain A	adapted	6.163	0.00093	4.000	14.714	0.000	2.132	0.000*	2.776
	nonadapted	5.820	0.00070						
Strain B	adapted	4.780	0.00520	4.000	3.238	0.016	2.132	0.032*	2.776
	nonadapted	4.563	0.00823						
Strain C	adapted	6.303	0.00253	3.000	2.884	0.032*	2.353	0.063	3.182
	nonadapted	6.070	0.01710						
Control	adapted	5.407	0.00503	2.000	27.721	0.001	2.920	0.001*	4.303
	nonadapted	4.223	0.00043						
Average	adapted	5.657	0.00093	4.000	11.759	0.000	2.132	0.000*	2.776
	nonadapted	5.397	0.00053						

Apple Cider

		Mean	Variance	df	t Stat	P(T<=t) one-tail	t Critical one-tail	P(T<=t) two-tail	t Critical two-tail
Strain A	adapted	5.997	0.00343	3.000	9.304	0.001	2.353	0.003*	3.182
	nonadapted	5.373	0.01003						
Strain B	adapted	4.460	0.00910	3.000	2.619	0.040*	2.353	0.079	3.182
	nonadapted	4.303	0.00163						
Strain C	adapted	5.480	0.01510	3.000	12.197	0.001	2.353	0.001*	3.182
	nonadapted	4.530	0.00310						
Control	adapted	4.013	0.00343	4.000	6.070	0.002	2.132	0.004*	2.776
	nonadapted	3.683	0.00543						
Average	adapted	5.233	0.00043	2.000	9.322	0.006	2.920	0.011*	4.303
	nonadapted	4.693	0.00963						

* These values are statistically significant ($P < 0.05$)

APPENDIX N
T-TEST RESULTS ASSESSING CROSS PROTECTION BY COMPARING ACID-ADAPTED WITH NONADAPTED AFTER BENZOATE/SORBATE TREATMENT (0.1%) USING RLU-DERIVED COUNT VALUES

E-Medium

		Mean	Variance	df	t Stat	P(T<=t) one-tail	t Critical one-tail	P(T<=t) two-tail	t Critical two-tail
Strain A	adapted	6.443	0.00023	3.000	119.678	0.000	2.353	0.000*	3.182
	nonadapted	4.187	0.00083						
Strain B	adapted	10.047	0.00263	4.000	38.276	0.000	2.132	0.000*	2.776
	nonadapted	8.087	0.00523						
Strain C	adapted	7.867	0.00163	3.000	148.328	0.000	2.353	0.000*	3.182
	nonadapted	3.730	0.00070						
Control	adapted	3.303	0.00163	3.000	8.601	0.002	2.353	0.003*	3.182
	nonadapted	2.850	0.00670						
Average	adapted	7.863	0.00043	2.000	77.133	0.000	2.920	0.000*	4.303
	nonadapted	4.757	0.00443						

Buffer

		Mean	Variance	df	t Stat	P(T<=t) one-tail	t Critical one-tail	P(T<=t) two-tail	t Critical two-tail
Strain A	adapted	3.210	0.00130	4.000	-54.609	0.000	2.132	0.000*	2.776
	nonadapted	4.620	0.00070						
Strain B	adapted	6.770	0.00190	4.000	40.638	0.000	2.132	0.000*	2.776
	nonadapted	5.457	0.00123						
Strain C	adapted	4.463	0.00823	2.000	19.749	0.001	2.920	0.003*	4.303
	nonadapted	3.427	0.00003						
Control	adapted	3.230	0.00040	3.000	34.092	0.000	2.353	0.000*	3.182
	nonadapted	2.077	0.00303						
Average	adapted	5.103	0.01373	4.000	13.919	0.000	2.132	0.000*	2.776
	nonadapted	3.813	0.01203						

Apple Cider

		Mean	Variance	df	t Stat	P(T<=t) one-tail	t Critical one-tail	P(T<=t) two-tail	t Critical two-tail
Strain A	adapted	4.480	0.00310	2.000	39.091	0.000	2.920	0.001*	4.303
	nonadapted	3.197	0.00013						
Strain B	adapted	5.927	0.00163	3.000	62.896	0.000	2.353	0.000*	3.182
	nonadapted	4.330	0.00030						
Strain C	adapted	3.987	0.00173	3.000	37.472	0.000	2.353	0.000*	3.182
	nonadapted	2.957	0.00053						
Control	adapted	3.250	0.00070	3.000	60.249	0.000	2.353	0.000*	3.182
	nonadapted	2.150	0.00030						
Average	adapted	4.670	0.00190	4.000	29.395	0.000	2.132	0.000*	2.776
	nonadapted	3.400	0.00370						

* These values are statistically significant ($P < 0.05$)

APPENDIX O
T-TEST RESULTS ASSESSING CROSS PROTECTION BY COMPARING ACID-ADAPTED WITH NONADAPTED AFTER FREEZE-THAW USING RLU-DERIVED COUNT VALUES

E-Medium

		Mean	Variance	df	t Stat	P(T<=t) one-tail	t Critical one-tail	P(T<=t) two-tail	t Critical two-tail
Strain A	adapted	2.473	0.01803	2.000	-1.025	0.207	2.920	0.413	4.303
	nonadapted	2.553	0.00023						
Strain B	adapted	2.150	0.00360	3.000	2.703	0.037*	2.353	0.074	3.182
	nonadapted	1.970	0.00970						
Strain C	adapted	2.917	0.00053	2.000	5.382	0.016	2.920	0.033*	4.303
	nonadapted	2.693	0.00463						
Control	adapted	2.330	0.00390	4.000	0.000	0.500	2.132	1.000	2.776
	nonadapted	2.330	0.00390						
Average	adapted	2.477	0.01083	2.000	0.054	0.481	2.920	0.962	4.303
	nonadapted	2.473	0.00043						

Buffer

		Mean	Variance	df	t Stat	P(T<=t) one-tail	t Critical one-tail	P(T<=t) two-tail	t Critical two-tail
Strain A	adapted	2.093	0.00173	4.000	-2.925	0.022	2.132	0.043*	2.776
	nonadapted	2.180	0.00090						
Strain B	adapted	1.950	0.00130	3.000	0.762	0.251	2.353	0.501	3.182
	nonadapted	1.917	0.00443						
Strain C	adapted	2.500	0.00280	3.000	2.133	0.061	2.353	0.123	3.182
	nonadapted	2.377	0.00723						
Control	adapted	2.157	0.00243	3.000	-1.151	0.167	2.353	0.333	3.182
	nonadapted	2.240	0.01330						
Average	adapted	2.157	0.00043	2.000	0.302	0.396	2.920	0.791	4.303
	nonadapted	2.143	0.00543						

Apple Cider

		Mean	Variance	df	t Stat	P(T<=t) one-tail	t Critical one-tail	P(T<=t) two-tail	t Critical two-tail
Strain A	adapted	2.120	0.00280	4.000	0.339	0.376	2.132	0.751	2.776
	nonadapted	2.103	0.00443						
Strain B	adapted	2.017	0.00703	3.000	3.564	0.019	2.353	0.038*	3.182
	nonadapted	1.820	0.00210						
Strain C	adapted	2.543	0.00043	4.000	19.622	0.000	2.132	0.000*	2.776
	nonadapted	2.173	0.00063						
Control	adapted	2.307	0.00343	2.000	8.449	0.007	2.920	0.014*	4.303
	nonadapted	2.003	0.00043						
Average	adapted	2.207	0.00063	2.000	3.181	0.043*	2.920	0.086	4.303
	nonadapted	2.020	0.00970						

* These values are statistically significant ($P < 0.05$)

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BIOGRAPHICAL SKETCH

Samir Kim Assar was born the son of Kirsten and Kamal Assar, and brother of Sami Assar in Kettering, Ohio, on March 12, 1970. He graduated from Lake Mary High School in 1988 to pursue a bachelor's degree in Biological Sciences at Florida State University. Immediately after his graduation from Florida State University in 1992, he participated in two 6-month dual externships with the Florida Department of Law Enforcement and in the Florida Department of Environmental Protection. In the Fall of 1994, Samir enrolled at the University of Florida as a Master's Student in the Department of Food Science and Human Nutrition where he studied under the auspices of Dr. Gary E. Rodrick who provided him with a 0.33-time research assistantship. During his Master's career, he was initiated into the honorary agricultural fraternity, Gamma Sigma Delta, and served as a Florida section IFT student representitive. He was accepted into a student career experience program by the United States Food and Drug Administration (USFDA) after his first two years as a Ph. D. candidate. To fulfill his appointment, Samir will be employed by the USFDA as a research scientist in the Center for Food Safety and Applied Nutrition (CFSAN).

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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